The effect of tocotrienols on vascular function

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Saher Fatima Ali

BSc (Hons) (Victoria University)

School of Health and Biomedical Sciences
College of Science, Engineering and Health
RMIT University

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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Saher Fatima Ali

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“Seek knowledge from the cradle to the grave.”

I dedicate this manuscript to my Papa; our dream has finally come true.

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Publications

1. Jason CD Nguyen, **Saher F Ali**, Sepideh Kosari, Owen L Woodman, Sarah J Spencer, Simon A Killcross and Trisha A Jenkins. (2017). Western diet chow consumption in rats induces striatal Fos expression while reducing dopamine levels independent of a spatial memory deficit in the radial arm maze. *Frontiers in Behavioural Science. Vol* 3. Issue 22*.*

2. **Saher F Ali**, Jason CD Nguyen, Tricia A Jenkins and Owen L Woodman (2016). Tocotrienol rich tocomin attenuates oxidative stress and improves endothelium-dependent relaxation in aortae from rats fed a high-fat western diet. *Frontiers in Cardiovascular Medicine. Vol* 3 *Issue 39.*

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Communications

1. **Saher F Ali**, Jason CD Nguyen, Trisha A Jenkins and Owen L Woodman (2016). Tocomin attenuates oxidative stress and improves NO mediated relaxation in high-fat fed western diet rat aorta. *Society for Redox Biology and Medicine*. California. Unites States of America

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4. **Saher F Ali**, Jason CD Nguyen, Trisha A Jenkins and Owen L Woodman (2014). Acute tocomin treatment improves endothelium-dependent relaxation in aortae from diabetic and western diet fed rats. *FASEB J*. Vol. 28 no. 1 Supplement April 2014. California. Unites States of America

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8. **SF Ali** & OL Woodman (2013). Tocomin restores endothelium-dependent relaxation in diabetic rat aorta. Biomed Link Melbourne. Australia

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TABLE OF CONTENTS

List of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Abbreviations

ACh = acetylcholine

AGE = advanced glycation end products

ATP = adenosine triphosphate

Akt = protein kinase B

BAT = brown adipose tissue

BGL = blood glucose levels

BH_4 = tetrahydrobiopterin

CaM = calmodulin

CAT = catalase

cav-1 = caveolin-1

cGMP = cyclic guanosine monophosphate

CVD = cardiovascular disease

DAG = diacylglycerol

DMSO = dimethyl sulfoxide

DPI = diphenyliodonium

EDH = endothelium-derived hyperpolarizing

EDHF = endothelium-derived hyperpolarizing factor

EDRF = endothelium-derived relaxing factor

eNOS = endothelial nitric oxide synthase

ETC = electron transport chain

FAD = flavin adenine dinucleotide

FFA = free fatty acid

FMN = flavin mono nucleotide

GTP = guanosine triphosphate

GSH = glutathione

GPx = glutathione peroxidase

G6PD = glucose-6-phosphate dehydrogenase

HbA1c = glycated haemoglobin

HRP = horseradish peroxidase

IK_{Ca} = intermediate calcium activated potassium channel

iNOS = inducible nitric oxide synthase

KPSS = high potassium physiological salt solution

L-NAME = N^G-Nitroarginine methyl ester

L-NNA = L-N^G-Nitroarginine

MDA = malondialdehyde

NADPH = nicotinamide adenine dinucleotide phosphate

NO = nitric oxide

nNOS = neuronal nitric oxide synthase

NOX = NADPH oxidases

$ONOO^-$ = peroxynitrite

PAGE = polyacrylamide gel electrophoresis

pAkt = phosphorylated protein kinase B

PE = phenlyephrine

pEC_{50} = $-\log_{10}$ concentration causing 50% response

PKC = protein kinase C

PPP = pentose phosphate pathway

RAGE = receptors for AGE

RNS = reactive nitrogen species

ROS = reactive oxygen species

R_{max} = maximum response

SD = standard diet

SDS = sodium dodecyl sulfate

SEM = standard error of the mean

Ser473 = serine 473

Ser1177 = serine 1177

Ser1179 = serine 1179

sGC = soluble guanylate cyclase

SK_{Ca} = small calcium activated potassium channel

SNP = sodium nitroprusside

SOD= superoxide dismutase

STZ = streptozotocin

T3 = tocotrienol

Thr 495 = threonine 495

TBST = tris buffered saline with tween-20

TC = tocopherol

TCA = tricarboxylic acid cycle

VSMC = vascular smooth muscle cell

WAT = white adipose tissue

WD = western diet

Abstract

Diabetes and obesity are non-communicable metabolic disorders that are reaching pandemic proportions across the world. Both diabetic and obese patients are at an increased risk of developing cardiovascular complications that are preceded by endothelial dysfunction. Vascular endothelium releases many chemicals such as the vasoconstrictor endothelin-1 and vasodilators such as prostacyclin and nitric oxide (NO). NO is synthesized by the enzyme endothelial derived nitric oxide synthase (eNOS). The bioavailability of NO has been demonstrated to be significantly reduced during diabetes and obesity due to increased oxidative stress in the vasculature that results from an increased production of reactive oxygen species (ROS) such as superoxide (O_2^-). Antioxidants have the potential to reduce oxidative stress in the vasculature. Vitamin E is a family of compounds, consisting of tocopherols and tocotrienols. Vitamin E, mainly as α-tocopherol, has been reported to have beneficial effects on vascular function through its antioxidant activity however large-scale clinical trials (mainly using α-tocopherol) have produced disappointing results.

There has been emerging literature indicating that tocotrienols, the less extensively studied members of the vitamin E family that share structural similarities with tocopherols possess comparatively superior antioxidant activity in comparison to tocopherols. Thus it has been proposed that tocotrienols may also have therapeutic benefits, including improvement of vascular endothelial function particularly in pathologies involving oxidant stress such as diabetes and obesity.

Due to the lack of literature that currently exists on the antioxidant effects of tocotrienols in the vasculature, the aim of this thesis was to examine the antioxidant activity of tocotrienols. We

also wanted to study the effect of a tocotrienol rich fraction of palm oil i.e. tocomin (composition: tocotrienols: 40%, α-tocopherol: 11% and palm olein: 38%) on endothelial function and O_2^- production in aortae in the presence of oxidative stress and in animal models of diabetes and obesity.

The first study, investigated whether tocotrienols, the less abundant components of vitamin E compared to tocopherols, might be more effective at preserving endothelial function. Superoxide generated by hypoxanthine/xanthine oxidase or rat aorta was measured using lucigenin-enhanced chemi-luminescence. The effect of α-tocopherol, α-, δ-, and γ-tocotrienols and a tocotrienol rich palm oil extract (tocomin) on levels of O_2^- was assessed. Endothelial function in rat aorta was assessed in the presence of the auto-oxidant pyrogallol. Whilst all of the compounds displayed antioxidant activity, the tocotrienols were more effective when O_2^- was produced by hypoxanthine/xanthine oxidase whereas tocomin and α-tocopherol were more effective in the isolated aorta. Tocomin and α-tocopherol restored endothelial function in the presence of oxidant stress but α-, δ-, and γ-tocotrienols were ineffective. The protective effect of tocomin was replicated when the tocotrienols were present with, but not without, α-tocopherol. Tocotrienol rich tocomin was more effective than α-tocopherol at reducing oxidative stress and restoring endothelium-dependent relaxation in rat aortae and although α-, δ-, and γ-tocotrienols effectively scavenged O_2^-, they did not improve endothelial function.

Having observed the antioxidant activity of tocomin and its ability to improve endothelial function in a pyrogallol-induced model of oxidative stress, the second study examined whether

the antioxidant effect of tocomin could be replicated in animal models of diabetes and obesity and the possible mechanisms of endothelial dysfunction in aortae from diabetic and obese rats. Diabetes was induced in Wistar rats by a single tail vein injection of streptozotocin (50 mg/kg). Rats remained diabetic for 10 weeks. Obesity was induced by feeding Wistar Hooded-rats a high-fat western diet (WD; 21% fat) for 12 weeks. Aortae from both diabetic and obese rats had increased oxidative stress that was seen as an increase in O_2^- production. Acute treatment of the aortae with tocomin was able to attenuate O_2^- production in both diabetic and WD (obese) rat aortae. This study also demonstrated that both diabetes and a high-fat western diet cause endothelial dysfunction in the diabetic and WD aortae. Diabetes reduced sensitivity to acetylcholine (ACh) and reduced the maximum response in the aorta, whereas in comparison a WD only reduced sensitivity to ACh while maximum relaxation was preserved. Endothelial dysfunction was attenuated acutely in the diabetic and WD rat aortae by the addition of α-tocopherol and tocomin, where tocomin was up to 100 times more potent than α-tocopherol as demonstrated in the previous study. We also demonstrated that there was a decrease in NO bioavailability in WD rat aortae in comparison to the standard diet (SD) aortae that correlates with endothelial dysfunction in the WD rat aorta.

The study also contrasted the different mechanisms of endothelial dysfunction in the diabetic and WD rat aortae in comparison to the sham and SD aortae respectively. Increased O_2^- production in the diabetic and WD aortae was attributed to an increase in the expression of the O_2^- producing enzyme Nox2. Both diabetic and WD aortae also had decreased eNOS expression. However during diabetes a decrease in eNOS expression was not associated with a decrease in the ratio of phosphorylated Akt to Akt (pAkt/Akt), calmodulin (CaM) or caveolin-1 (cav-1) expression suggesting that in advanced diabetes (10-weeks) pAkt/Akt, cav-1 or CaM do not provide any compensatory mechanism to improve endothelium-dependent relaxation

and perhaps the up-regulation of other O_2^- producing pathways e.g. polyol pathway and mitochondrial activity may cause uncoupling of eNOS thus further exacerbating endothelial dysfunction by impairing eNOS function. In contrast in the obese aorta in the presence of oxidative stress, decreased eNOS expression was coupled with a decrease in the ratio of pAkt/Akt and of CaM expression. There was also an increase in cav-1 expression indicating that a decrease in the expression of positive eNOS regulatory proteins (pAkt/Akt and CaM) and an increase in the expression of the negative eNOS regulatory protein cav-1 in the obese rat aortae was associated with endothelial dysfunction during obesity.

Having established that tocomin could acutely improve endothelial function *in vitro* the next aim was to investigate whether the beneficial effects could also be observed after treatment *in vivo* in the diabetic and obese rats. The diabetic and obese animal models previously described were used in this study again where diabetic rats were treated with tocomin (40mg/kg/day s.c.) 6 weeks into the study and the obese rats commenced tocomin treatment (40 mg/kg/day s.c.) 8 weeks into the study. Both groups were treated for a period of 4 weeks. Tocomin did not affect the diet-induced weight loss in the diabetic rats or weight gain or the increase in epididymal fat of the obese rats. Also similar to the previous study, both the diabetic and obese rat aortae had increased O_2^- production that was associated with increased Nox2 expression. Four week treatment of both diabetic and obese rats with tocomin decreased vascular O_2^- production. The decrease in O_2^- production was correlated with decreased expression of the vascular NADPH oxidase subunit Nox2. The diabetic rat aortae also had an impaired maximum response and the obese rat aortae had a decreased sensitivity to ACh at the end of the experimental period. Four week treatment of diabetic and obese rats with tocomin significantly improved endothelial function which is due to an increase in NO bioavailability.

Other beneficial actions of tocomin to reduce vascular oxidative stress that was observed in this study included an increase in the expression of eNOS and the eNOS activity promoting proteins CaM and pAkt/Akt in the obese rat aortae. Further, there was decreased expression of the inhibitory protein caveolin-1 in the obese rat aortae. Neither diabetes nor tocomin had any affect on pAkt/Akt, CaM and cav-1 expression.

In conclusion, this thesis provides further insights in the pathological process underlying endothelial dysfunction in the macro-vasculature over the duration of diabetes and obesity. Tocomin that is a mixture of tocotrienols and α-tocopherol is up to 100 times more effective than α-tocopherol or tocotrienol isomers alone where the presence of α-tocopherol is necessary for the efficacy of tocomin. The beneficial actions of tocomin in this diet-induced model of diabetes and obesity suggest that it may have potential to be used as an adjunct therapeutic with other anti-diabetic and anti-obesigenic drugs such as metformin and sibutramine to prevent vascular disease in diabetes and obesity.

Chapter 1

Introduction.

CHAPTER 1: INTRODUCTION

Diabetes is a complex and progressive metabolic disease that is classically defined as a chronic state of hyperglycaemia and disturbances to carbohydrate, protein and fat metabolism due to a marked reduction in the production or efficacy of the hormone insulin or both (Alberti and Zimmet, 1998). There are 3 types of diabetes; type 1, gestational and type 2. Type 1 diabetes, also known as juvenile diabetes, has an early onset of around 12 years of age and requires lifelong management with insulin injections due to an autoimmune destruction of the β-pancreatic cells that consequently leads to a loss of insulin release (Harrison et al., 2008). Gestational diabetes occurs during pregnancy and has very similar clinical characteristics to type 2 diabetes. Gestational diabetes usually disappears after childbirth however women who have had gestational diabetes have a higher risk of developing type 2 diabetes and metabolic syndrome later during their life (Singh et al., 2013a). Type 2 diabetes, formerly known as non-insulin dependent diabetes, is the most common form of diabetes, accounting for almost 90% of all diabetic patients worldwide (Craig et al., 2007). Type 2 diabetes has a late age of onset, occurring mainly in people over the age of 40 years, and is preceded by insulin resistance. Type 2 diabetes is strongly correlated with obesity and a sedentary lifestyle (Beck-Nielsen et al., 1995).

Diabetes is reaching epidemic proportions not just in Australia but also worldwide. There are currently 346 million people worldwide with diabetes and it is projected by the World Health Organisation (WHO) that between 2005 and 2030 diabetes related deaths will double (WHO, 2013). Diabetes is a significant health burden in Australia where it has an estimated conservative cost of $3.1 billion to the Australian government per year

(Colagiuri et al., 2011). Also, due to the increasing incidence of childhood obesity worldwide, type 2 diabetes is now starting to occur in children at alarming rates. In Australia 11% of all type 2 diabetes cases occur in children between the ages of 10–18 years (Craig et al., 2007).

Diabetic patients are at higher risk of developing pathologies such as diabetic retinopathy, nephropathy, neuropathy and cardiovascular disease (CVD) (Ali and Maron, 2006, Pasaoglu et al., 2004). CVD is the leading cause of death of diabetic patients in Australia (Ali and Maron, 2006). Diabetic patients have twice the risk of developing CVD compared to their healthy counterparts. It is estimated that in Australia more than two-thirds of those who die of CVD had diabetes or pre-diabetes five years earlier (Craig et al., 2007). It is due to these statistics, and similar statistics worldwide, that diabetes has become a major health priority for the Australia and other nations (Buse et al., 2007).

As mentioned previously obesity is a major risk factor for type 2 diabetes. The WHO defines obesity as abnormal or excessive fat accumulation in the body that may impair health with a body mass index (BMI) greater than 30 kg/m^2. A BMI between 25-29 is considered overweight. Obesity is more prevalent than diabetes where in 2014 more than 1.9 billion adults worldwide, 18 years and older, were overweight (WHO, 2015). Of these, over 600 million were obese. Obesity is also becoming increasingly prevalent amongst children worldwide, where in 2013, 42 million under the age of 5 were obese. (WHO, 2015). From 2007-2008 it was estimated in the US that 31.6% of children were overweight and 16.9% of children were obese (Cote et al., 2013). Statistics for Australia are very similar where 63% of Australian adults are overweight or obese and 25% of Australian

children are either overweight or obese. Obesity is also the second biggest contributor to the burden of disease in Australia (Australian Institute Welfare Health, 2015).

Similarly to diabetic patients, obese patients are also at a very high risk of developing cardiovascular related complications such as hypertension, myocardial infarction and stroke. There is substantial literature that consistently associates endothelial dysfunction arising from oxidative stress, or free radical mediated damage, with diabetes, obesity and CVD related pathologies (Stephens et al., 2008). This review will discuss the physiology of the vascular endothelium and oxidative stress during diabetes and obesity, proposed mechanisms through which oxidative stress is increased during diabetes and obesity and how it may cause endothelial dysfunction and initiate cardiovascular related pathology and CVD. This review will also consider the possible therapeutic use of antioxidants with a major focus on α-tocopherol and to a lesser extent tocotrienols that have not been extensively studied. The biological actions of these compounds and their potential use to prevent diabetes and obesity-induced CVD are also reviewed.

1.1 Blood vessels and endothelium

Blood vessels are dynamic structures that possess an endothelium consisting of, a thin monolayer layer of cells lining the lumen. Endothelium is the continuum of the endocardial layer of the heart and internally lines all blood vessels (Figure 1.1). The endothelium is a semi-permeable membrane and has several functions such as controlling vascular tone by releasing potent vasodilators and anti-platelet agents, prostacyclin and nitric oxide (NO) (Cahill and Redmond, 2016). The endothelium also releases vasoconstrictors that oppose the actions of the vasodilators such as NO. Endothelin-1, and angiotensin II are known to be released from the endothelium to cause vasoconstriction

(Sena et al., 2013, Tirapelli et al., 2009). Vascular endothelium also controls the selective adhesion and emigration of leukocytes from the bloodstream and it is also a source of the glycoprotein Von Willebrand factor which is important in coagulation (Pearson, 2000). This suggests that the endothelium possesses anti-atherosclerotic and antithrombotic properties. An important function of the endothelium that is of major interest is the regulation of vascular tone (Sydow and Münzel, 2003). This is achieved by the release of various endogenous substances which cause vasorelaxation/ constriction e.g. NO, prostacylin and an unidentified endothelium-derived hyperpolarising factor (EDHF) (Félétou and Vanhoutte, 2004, Furchgott, 1983). Vasoactive substances have been shown to act in an endothelium-dependent or independent manner. For example, acetylcholine (ACh) and bradykinin, amongst other endogenous vasodilators act on the endothelium to release factors causing vascular smooth muscle cell relaxation. By contrast NO donors such as sodium nitroprusside (SNP) or arachadonic acid act directly on vascular smooth cells to cause relaxation i.e. in an endothelium-independent manner.

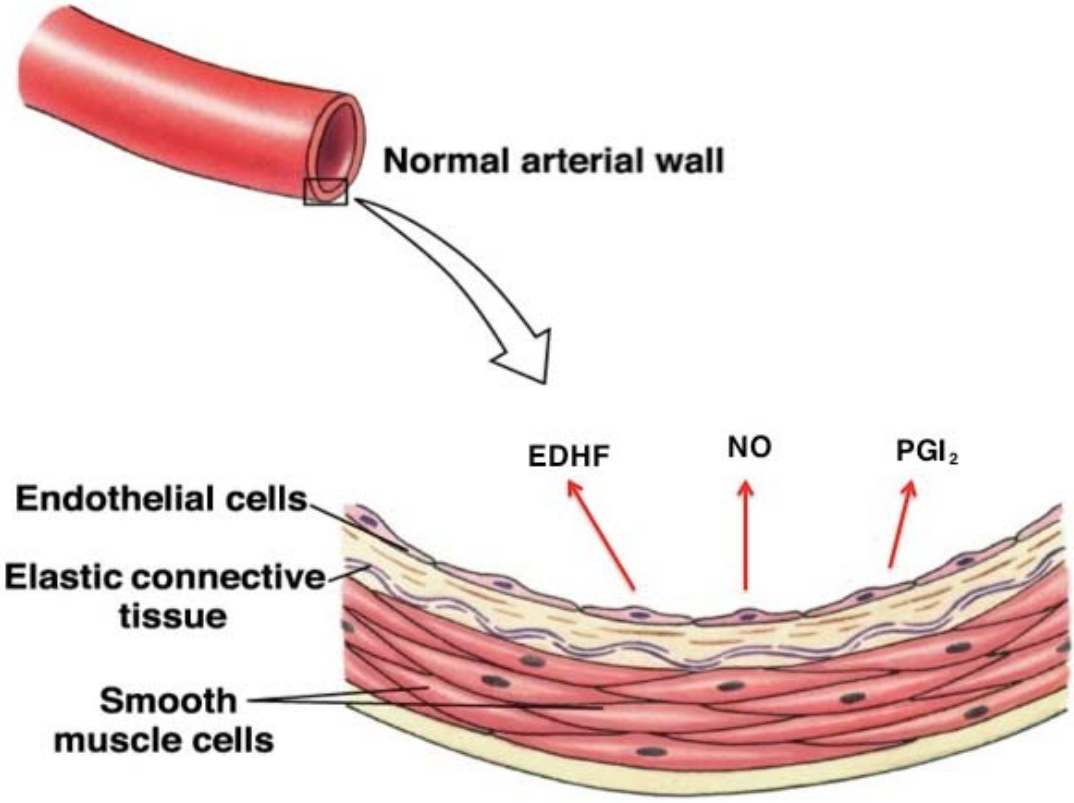


Figure 1.1 The structure and function of the endothelium: The endothelium is the innermost lining on the lumen of an artery that is the continuum of the endocardial layer of the heart. Endothelium allows for gas exchange and produces the potent vasodilator NO amongst other vasodilators such as prostacyclin and endothelium-derived hyperpolarising factor (EDHF). *Image adapted from (HeartSense, 2015).*

1.2 Endothelium-dependent relaxation

Endothelium-dependent relaxation in the vasculature is dependent on the bioavailability of endothelium derived relaxing factors (EDRF), the most important of which is NO (Moncada et al., 1991). NO is a gaseous free radical with a physiological half life of 4-6 seconds (Moncada et al., 1991). In 1980 it was demonstrated by Furchgott and Zawadzki (Furchgott and Zawadzki, 1980) that an unknown EDRF is released from the vascular endothelium in response to muscarinic agonists. By 1989 it was demonstrated by Ignarro et al. (1987) and Moncada et al. (1988) individually that the potent endothelium-derived vasorelaxant is in fact NO with cardiovascular signalling properties (Yetik-Anacak and Catravas, 2006). NO also possesses several other functions including; at physiological concentrations (100 pM- 5 nM) it has neurotransmitter and immunological functions (Hall and Garthwaite, 2009). NO can also be cytotoxic and induce DNA damage and lipid peroxidation (Wink and Mitchell, 1998, Moncada et al., 1991) when reacting with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) (Burney et al., 1999). Also since the discovery of NO, other EDRF's and potential endothelium-derived hyperpolarising factors (EDHF) have been identified e.g. potassium (K^+), hydrogen sulfide (H_2S) and prostacylin (PGI_2) (Félétou et al., 2011, Triggle and Ding, 2010, Wang, 2009).

NO is synthesized from a group of enzymes known as nitric oxide synthase (NOS) (Rafikov et al., 2011). There are three known isoforms of NOS; type I neuronal NOS (nNOS; synthesize NO as a neurotransmitter from neurons), type II inducible NOS (iNOS; synthesize NO from macrophages and cytokines) and type III endothelial

derived nitric oxide synthase (eNOS; synthesize NO from vascular endothelial cells) (Treuer and Gonzalez, 2015). eNOS derived NO synthesis is stimulated in the body in response to various stimuli such as ACh, bradykinin, serotonin and shear stress (Arnal et al., 1999).

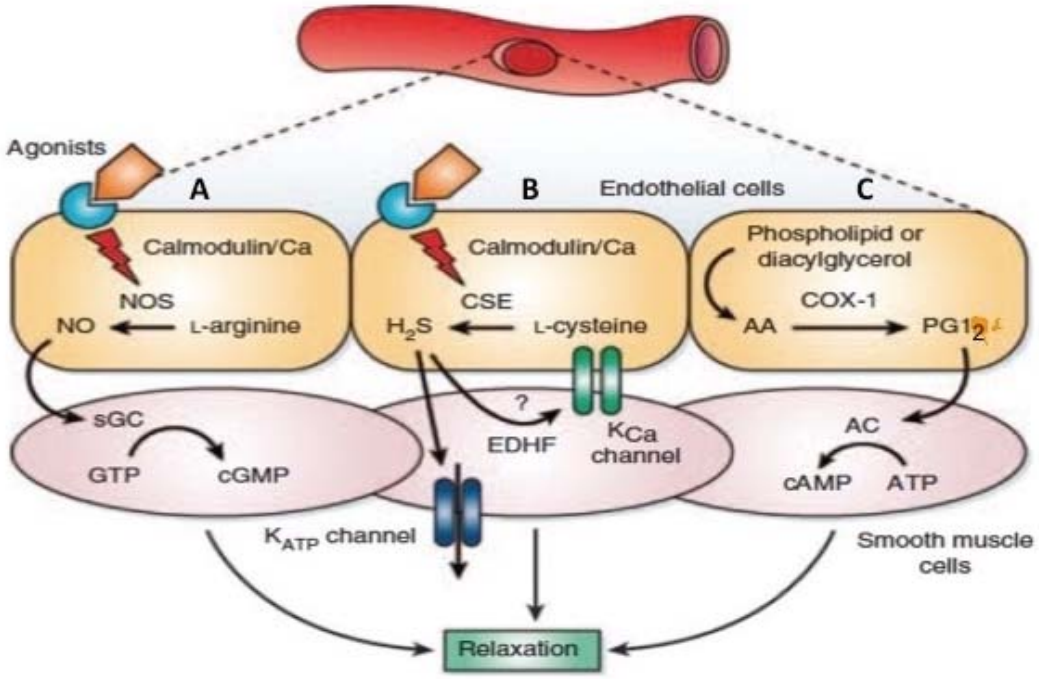


Figure 1.2 Endothelium-dependent relaxation: Agonists bind to their respective receptors on endothelial cells to stimulate NO synthesis (A) eg: ACh/bradykinin stimulate NO synthesis from the conversion of L-arginine to L-citrulline that is catalysed by eNOS. NO then diffuses through the vascular smooth muscle cell and activates soluble guanylate cyclase (sGC) which catalyses the conversion of GTP into cGMP. This causes vasorelaxation by calcium re-entering the sacrcoplasmic reticulum. (B) H_2S is produced in endothelial cells from the enzyme cystathionine γ-lyase (CSE) and activates ATP sensitive potassium channels (K_{ATP}) and causes vasorelaxation. (C) Cyclooxygenase-1 (COX-1) catalyse the conversion of arachadonic acid (AA) into PGI_2 to diffuse into the vascular smooth muscle to activate cAMP to cause vasorelaxation. *Adapted from Wang (2009).*

These stimuli cause an increase in the endothelial free intracellular calcium (Ca^{2+}) concentration which subsequently causes the binding of eNOS to calmodulin (CaM),

the regulatory protein of eNOS (Mariotto et al., 2004, Arnal et al., 1999). eNOS catalyses the conversion of the amino acid L-arginine to L-citrulline via a series of complex chemical reactions to produce NO (Alkaitis and Crabtree, 2012). NO then reaches the vascular smooth muscle cells via diffusion and activates the enzyme soluble guanylate cyclase (sGC) (Figure 1.2). sGC catalyses the conversion of GTP to cGMP which then causes endothelium-dependent relaxation via Ca^{2+} reuptake into the sarcoplasmic reticulum (Katsuki et al., 1977). Other endogenous substances that are capable of mediating endothelium-dependent relaxation include prostacyclin and endothelium derived hyperpolarising (EDH)-type relaxation. The mechanism through which NO and other EDRF's cause endothelium-dependent vasorelaxation is illustrated in Figure 1.2.

1.3 eNOS and its regulators

NO is a free radical possessing antioxidant properties that can be highly toxic at high concentrations. It has a very short half-life of 4-6 seconds and has a high affinity to react with O_2^- to form $ONOO^-$ (Moncada et al., 1991). As mentioned previously nitric oxide synthases (NOSs) are a group of enzymes responsible for the production of NO and eNOS is responsible for the production of NO in the vasculature. All NOS isoforms are dimerized proteins with an oxygenase and reductase domain separated by a calmodulin (CaM) binding domain (Figure 1.3) (Braam and Verhaar, 2007, Mariotto et al., 2004). This review will focus on type III NOS i.e. eNOS.

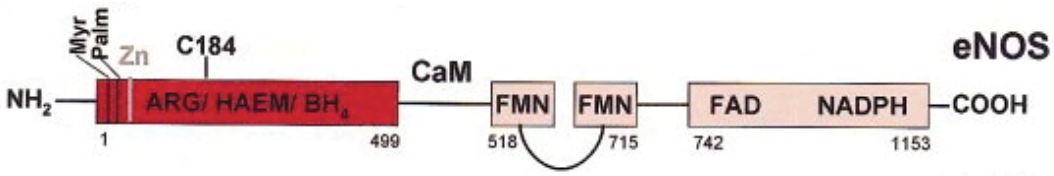


Figure 1.3 Simplified eNOS structure: eNOS is a dimeric protein consisting of two domains with binding sites for L- arginine, NADPH, FAD, FMN, iron and BH_4. NO production is stimulated when the regulatory protein calmodulin (CaM) binds to eNOS. *Adapted from Alderton et al. 2001*.

1.3.1 eNOS and calcium-dependent regulation of eNOS

As mentioned previously, eNOS is a dimeric enzyme that has a mass of approximately 260kDa and consists of approximately 1203 amino acids and. eNOS has binding sites for several molecules which allow it to synthesize NO effectively. eNOS has a reductase domain containing binding sites for the cofactors NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and the oxygenase domain contains binding sites for heme, tetrahydrobiopterin (BH_4), and L-arginine (Figure 1.3).

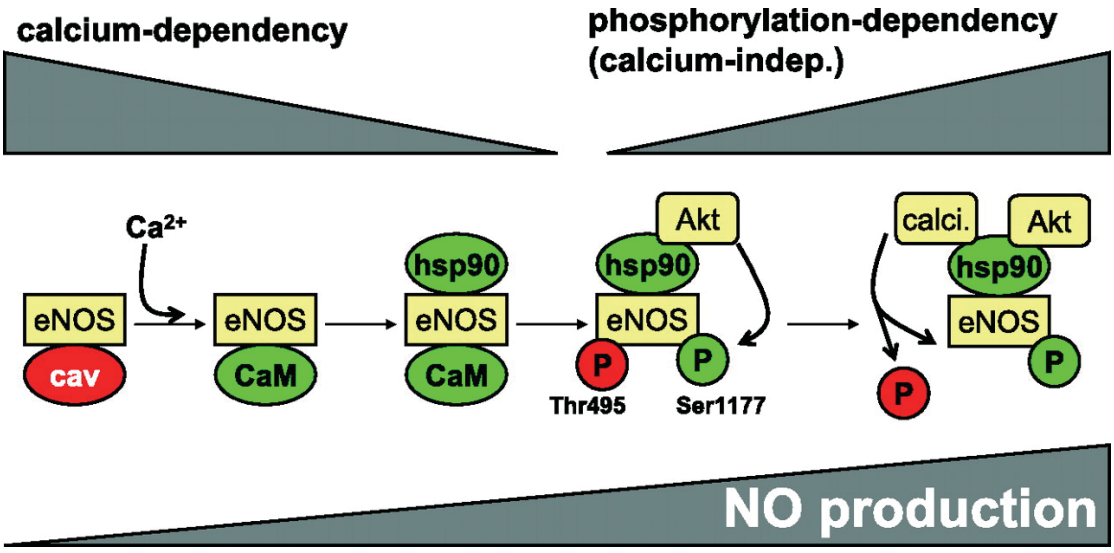
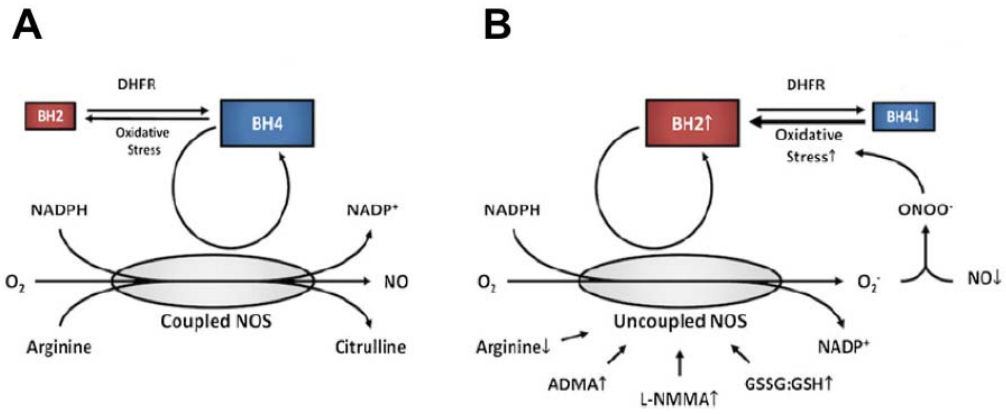


Figure 1.4: Schematic eNOS/Akt interaction: During resting conditions eNOS is in an inactive state bound to the cav-1 protein. An increase in intracellular Ca^{2+} concentration causes cav-1 to dissociate from the eNOS enzyme subsequently allowing CaM to bind to eNOS. This is followed by hsp90 binding to eNOS that activates Akt to phosphorylate eNOS at the s1177 site. The calcineurin (calci) protein dephosphorylate eNOS at the Thr495 site which is the inhibitory protein of eNOS. This *Taken from Baligand et al. (2009)* .

In its resting state, eNOS is bound to caveolin-1 (cav-1). Intracellular Ca^{2+} concentrations can increase in response to various agonists such as ACh and bradykinin or shear stress. This causes the dissociation of cav-1 from eNOS allowing CaM and heat shock protein 90 (hsp90) to bind to eNOS (Figure 1.4) in response to the elevated intracellular Ca^{2+} concentrations (Dudzinski and Michel, 2007). This complex causes protein kinase B (Akt) to phosphorylate eNOS at the serine 1177 site in humans (Ser1177) or Ser1179 in animals (Zhao et al., 2015) and dephosphorylate eNOS at the inhibitory site of threonine 495 (Thr495) (Balligand et al., 2009). Once eNOS is phosphorylated by Akt at Ser1177/1179 (Figure 1.4), NO synthesis commences through a five-electron oxidation of amino acid L-arginine producing NO and L-citrulline (Figure 1.5) (Alkaitis and Crabtree, 2012). Because an increase in intracellular

Ca^{2+} is crucial for eNOS activation, this process is called Ca^{2+}-dependent eNOS phosphorylation. eNOS can also synthesize NO in the absence of Ca^{2+}-CaM activation from dynamic shear stress and hormones (Zhao et al., 2015). This process is called Ca^{2+}-independent eNOS activation (Flemming et al., 1998).



ADMA: asymetrical dimethylarginine

LNMMA: NG-monomethyl-L-arginine

BH_2: dihydrobiopterin

DHFR: dihydrofolate reductase

Figure 1.5: eNOS derived NO production and the effect of uncoupled eNOS on NO production: (A) eNOS is stimulated when intracellular calcium binds to CaM that stimulates the conversion of L-arginine to L-citrulline producing NO as a by-product. (B) Increased oxidative stress and eNOS inhibitors such as ADMA and L-NMMA which are arginine analogues decrease the bioavailability of eNOS cofactors such BH_4 and NADPH that leads to uncoupling of the eNOS dimer and the production of O_2^- instead of NO. *Taken from Alkaitis and Crabtree (2012)* .

The bioavailability of eNOS cofactors is crucial to the proper functioning of eNOS. In disease conditions such as diabetes there is decreased bioavailability of cofactors such as BH_4 and NADPH (Chen et al., 2008). This can lead to uncoupling of the homodimer into eNOS monomers. In this state eNOS may start producing O_2^- rather than NO (Figure 1.5) (Satoh et al., 2005). This has been demonstrated to occur in disease states such as diabetes (Joshi and Woodman, 2012, Leo et al., 2011a). An excess of reactive

oxygen species (ROS) causes oxidative stress that is a very important component in the pathophysiology of endothelial dysfunction. Endothelial dysfunction eventually can become a major cause of cardiovascular related complications in diabetes and obesity (Della Rocca and Pepine, 2010).

1.4 Oxidative stress, endothelial dysfunction and cardiovascular disease

Oxidative stress is classically defined as "a disturbance in the prooxidant-antioxidant balance in favour of the former" (Sies, 1985). Oxidative stress in a cell can be due to either; a) an increase in the number of ROS and reactive nitrogen species (RNS) mainly consisting of free radicals which leads to an overload of the antioxidant defense system, b) a decrease in the number of antioxidant molecules either endogenous (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) or exogenous (vitamins C and E) leading to an imbalance of the redox system of a cell or c) a combination of both (Marks et al., 1996). Antioxidants are molecules that at low concentrations can prevent oxidation of various cell components by donating an electron to free radicals and that can initiate or take part in increasing the oxidative capacity of a cell (Nwose et al., 2007). However, after donating its electron, the antioxidant itself becomes a free radical (Nwose et al., 2007). This radical antioxidant may potentially oxidise cell components if it cannot be paired with another free radical, or find another molecule to donate its electron to, however due to their high chemical stability and low reactivity, antioxidant radicals are not chemically damaging and are recycled for reuse eg: vitamin C is involved in the recycling of vitamin E (Nwose et al., 2007, Maxwell and Lip, 1997).

ROS and RNS are highly reactive oxygen and nitrogen containing molecules that often have an unpaired electron in their atomic structure e.g. O_2^-, the hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$) (Figure 1.6) (Stephens et al., 2008). ROS and RNS can cause tissue damage through oxidation of various cellular components including DNA, membrane protein and lipids (Brownlee, 2005).

Endogenous antioxidant enzymes that are involved in ROS metabolism include CAT and GPx that converts H_2O_2 into H_2O and O_2. GPx and CAT share common cofactors with eNOS e.g. NADPH is reduced to $NADP^+$ by GPx to convert H_2O_2 into H_2O (Figure 1.7). During diseases where oxidative stress is high e.g. diabetes and obesity, the bioavailability of cofactors such as NADPH can be reduced due to NADPH being diverted to other chemical pathways e.g. the polyol pathway during diabetes. This reduced bioavailability of cofactors can compromise GPx and CAT activity and can further exacerbate oxidative stress (Nwose et al., 2007, Dincer et al., 2002). This can also compromise NO production due to the decreased bioavailability of NADPH.

Increased O_2^- production has been implicated in the pathophysiology of endothelial dysfunction which is a precursor to diabetes and obesity related cardiovascular complications (Rains and Jain, 2011). O_2^- in high concentrations can stimulate other cellular reactions for example, O_2^- reacting with other molecules such as NO to form $ONOO^-$, a highly toxic RNS (Figure 1.6) thus decreasing NO bioavailability. $ONOO^-$ can react with BH_4 to form dihydrobiopterin (BH_2) leading to the depletion of BH_4 (Alp and Channon, 2004) which is an important cofactor for eNOS to produce NO. This will consequently decrease the bioavailability of NO resulting in impairment of endothelium-dependent relaxation due to a decreased activation of sGC. eNOS activity

may subsequently decrease. BH_4 depletion is also known to cause uncoupling of eNOS from a dimeric structure into a monomeric structure (Chen et al., 2008) and as previously mentioned eNOS in its uncoupled structure produces O_2^- (Figure 1.4) (Satoh et al., 2005) that further impairs endothelium-dependent relaxation due to the lack of NO, thus making O_2^- production a self perpetuating process.

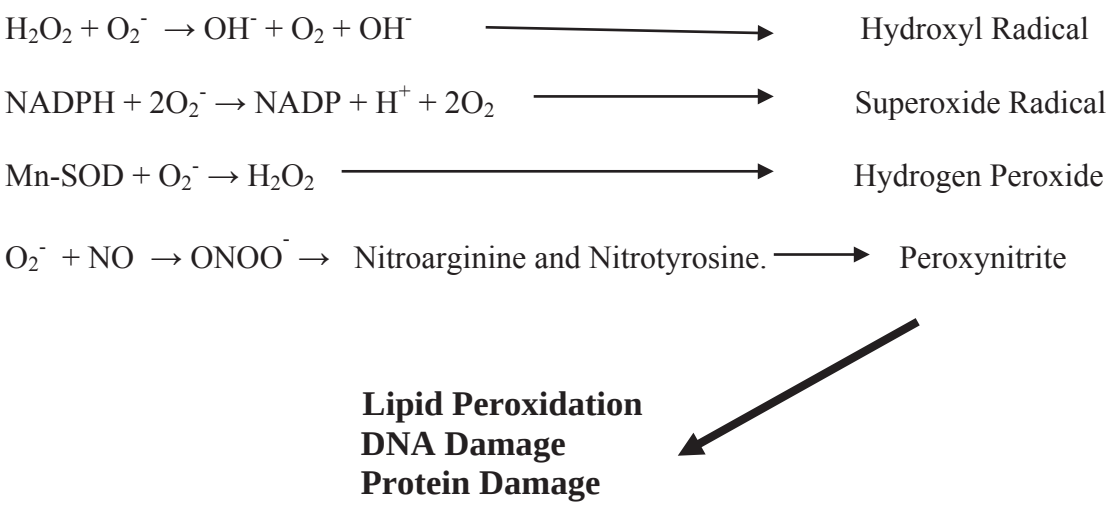


Figure 1.6 Potential sources of oxidative stress and its consequences: There are several physiological reactions that constantly occur to generate ROS and RNS such as oxidative phosphorylation and the pentose phosphate pathway (PPP). If ROS and RNS cannot be converted by antioxidants it can lead to oxidative damage in the form of membrane and DNA damage. *Adapted from Shah et al. (2007).*

Although ROS are produced in all cells as a by-product of a variety of physiological reactions, it is ultimately the lack of a cells capacity to convert ROS into a more stable molecule that increases the level of oxidative stress. Thus antioxidants provide cells with stability and membrane integrity through an antioxidant sink consisting of several endogenous enzymatic antioxidants e.g. SOD, CAT and GPx and exogenous non-enzymatic molecules e.g. vitamins C and E.

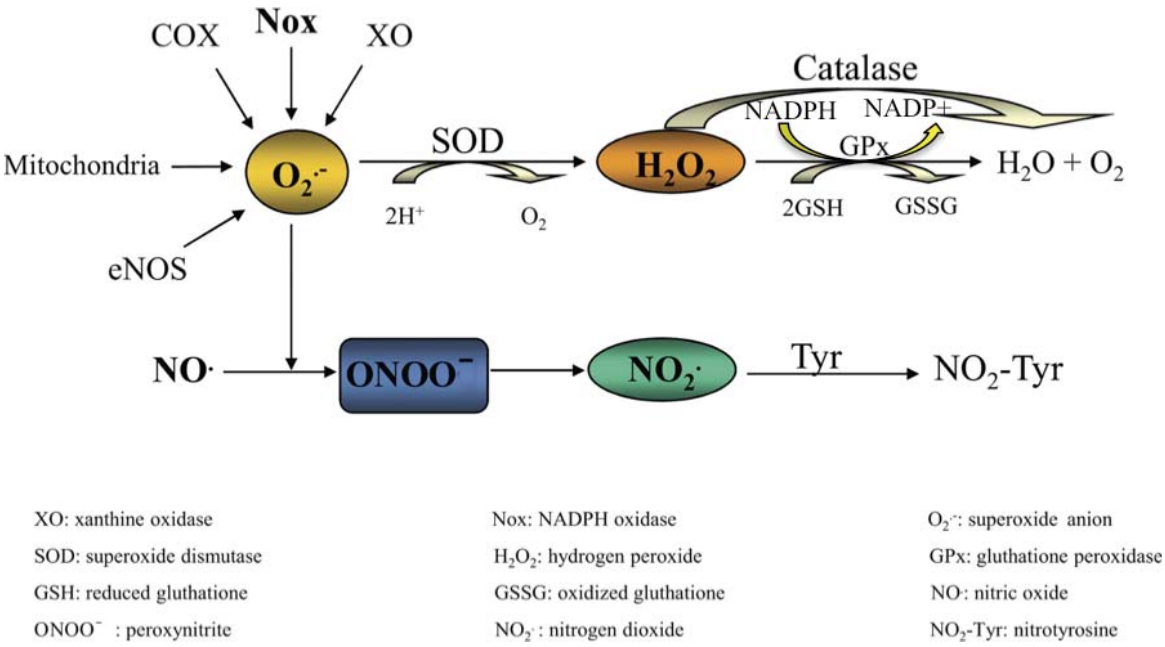


Figure 1.7 Sources of O_2^- and their metabolism: O_2^- can be produced from eNOS, Nox, COX and XO. O_2^- is converted into cytotoxic H_2O_2 by SOD. H_2O_2 is then converted into H_2O and O_2 by CAT and GPx. If GPx and CAT enzyme systems are saturated then ROS levels may increase. O_2^- also has a high affinity for NO and if enzymes such as SOD are compromised O_2^- can form $ONOO^-$ upon reacting with NO that can damage DNA. *Adapted from Frazziano et al. (2012)* .

1.5 Endothelial dysfunction

During diabetes, as a result of hyperglycaemia, and in obesity, due to an increased energy influx, there is a significant increase in O_2^- production due to mechanisms that will be discussed later (Antoniades et al., 2009). This increase in oxidative stress can subsequently lead to impaired endothelium-dependent relaxation resulting from decreased NO bioavailability or a lack of NO production resulting from uncoupling of the eNOS enzyme (Marinou et al., 2009).

1.5.1 Diabetes and CVD.

During diabetes O_2^- production becomes a vicious process that can cause damage to DNA and proteins, subsequently causing activation of inflammatory processes and monocyte recruitment in the arterial wall, which play an important role in the pathogenesis of atherosclerosis (Schaffer et al., 2012). Decreased NO bioavailability during diabetes causes endothelial dysfunction that promotes increased expression of cell adhesion molecules, increases platelet activation, impairs vascular relaxation and stimulates monocyte and macrophage migration (Penckofer et al., 2002). These processes play a major role in the pathogenesis of CVD (Schaffer et al., 2012). Hence, endothelial dysfunction is a central mechanism in the development of CVD related pathology during diabetes. Therefore it has been proposed that during diabetes oxidative stress may be alleviated with the use of appropriate therapy i.e. antioxidants, endothelium function can be improved, consequently delaying the onset of diabetes related CVD.

1.5.2 Obesity and CVD.

Obesity is a disease that is usually defined as a body mass index (BMI) greater than 30 kg/m^2. It is diagnosed by an accumulation of excess visceral body fat mostly in the abdominal area, and generally occurs as a result of the consumption of excess kilojoules (kJ) leaving the body with a positive energy balance i.e. energy consumption exceeding energy metabolized (Figure 1.8) (Hawkesworth, 2013).

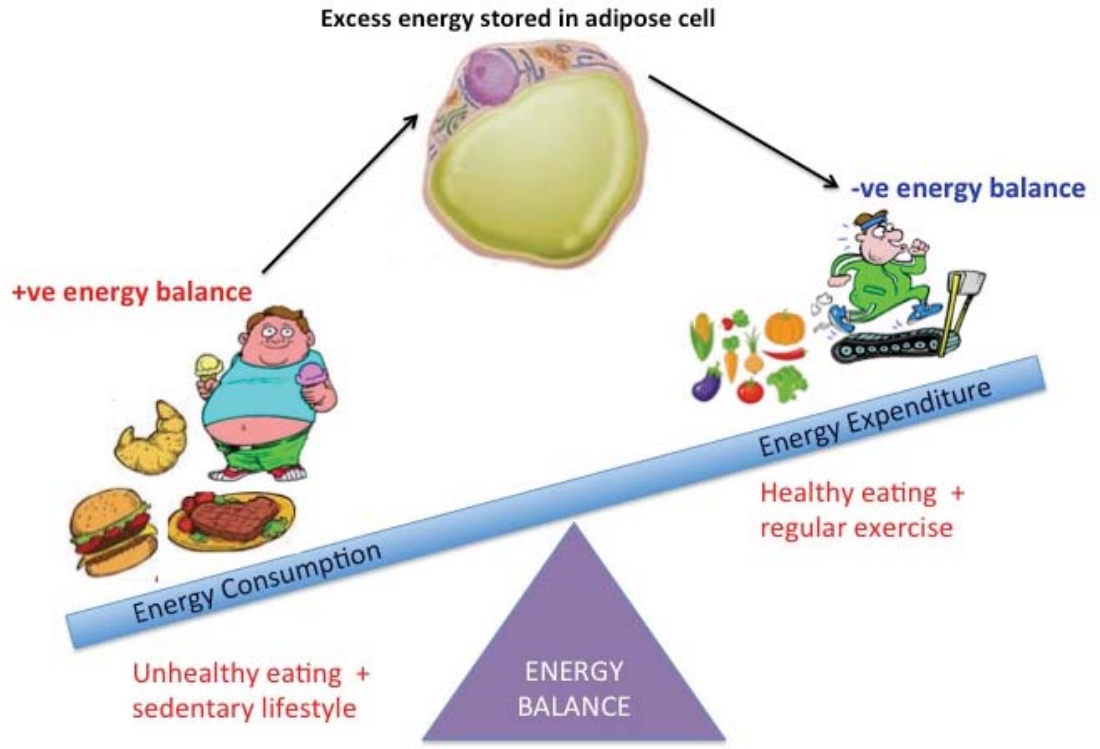


Figure 1.8 Obesity: Obesity occurs due to energy consumption exceeding energy expenditure which results in a positive energy balance and the accumulation of fat in adipocytes especially in the abdominal area. A healthy lifestyle incorporating exercise can maintain energy balance or have a negative energy balance that can lead to weight loss.

Energy in the body is derived from carbohydrate, protein or fat. Of these fat is the most energy dense (37 kJ/g) whereas carbohydrate and protein are the least energy dense (17 kJ/g). Carbohydrate, protein and fat are converted into glucose that is metabolised through the Krebs (tricarboxylic acid) cycle and electron transport chain. The product of glucose metabolism is adenosine triphosphate (ATP) which is the form in which energy is metabolised by the body during physiological reactions to convert stored energy into heat and kinetic energy (muscle contraction). When the total energy consumed in a day is not "burnt" (metabolised i.e. converted into kinetic energy through exercise or heat through bodily physiological reactions) it is converted into fat and stored in white adipose tissue (Matsuda and Shimomura, 2013). It is important to note

that although the cumulative effect of a positive energy balance is the most common cause of obesity, there are pathologies that may cause energy imbalances. These can include genetic and physiological diseases (i.e. Hashimoto disease/hypothyroidism), psychological factors (i.e. binge eating disorders, comfort eating), socioeconomic factors (limited nutritional information, limited finances to access healthy food), iatrogenic causes (antipsychotic medications e.g. clozapine) and physical factors (osteoarthritis or having restricted mobility) and rare genetic defects. Thus indicating obesity is a direct result from the interaction between the external and physiological milieu (Sansbury and Hill, 2014).

There are two types of adipose tissue in the body i.e. brown adipose tissue (BAT) and white adipose tissue (WAT). Brown adipose tissue is more abundant in newborns and its primary function is to protect newborns from heat loss by insulating the body. WAT is loose connective tissue that mainly consists of adipocytes (Figure 1.9). Adipocytes are composed of cholesterol esters and triglycerides. The primary function of WAT is to serve as a reservoir of energy that can be metabolised when required. It is mainly found around visceral organs where its main function is insulation and to cushion important organs from external shock. WAT is also an important endocrine organ producing oestrogen, leptin (satiety hormone) and in excess of 260 peptides which will not be covered in this thesis (Mutt et al., 2014). Adipocytes also express receptors for insulin, growth hormone and the stress hormone cortisol (Schaffler et al., 2006). Adipocytes of WAT have the capacity to expand up to 1000 times their original size and are significantly increased in mass during obesity (Jo et al., 2012). WAT mass is what is attempted to be "*lost*" during any weight loss regime.

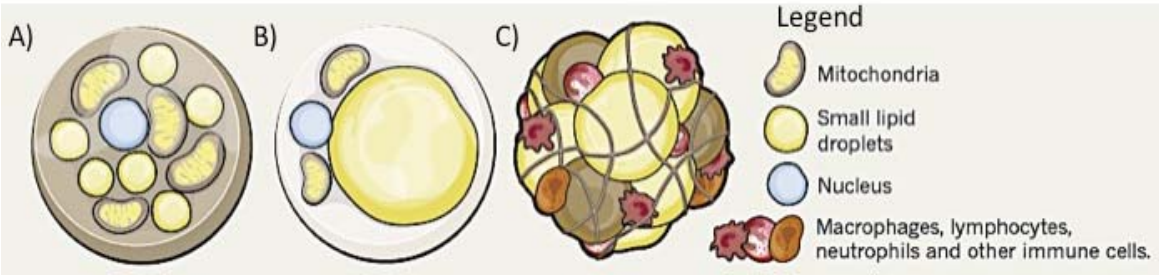


Figure 1.9 White and brown adipose tissue**:** Brown adipose tissue (A; BAT) has a high oxidative capacity due to possessing a large number of mitochondria and its main function is to insulate. White adipose tissue (B; WAT) volume is occupied by fat and its function is to store energy in the form of fat. WAT has very small oxidative capacity due to the low number of mitochondria in contrast to BAT. During obesity (C) WAT becomes infiltrated with macrophages and lymphocytes due to increased fat volume. This places WAT in a state of inflammation that leads to adipocyte apoptosis and the initiation of atherosclerotic processes in the vasculature. *Adapted from Owens (2014).*

During obesity, due to a significant positive energy balance and the accumulation of fats, there is an increase in free fatty acids (FFA) circulating in the blood. These FFA's undergo β-oxidation that, during metabolism in the mitochondria, produce O_2^- at very high rates. This causes excessive stress on the endogenous antioxidant GPx and CAT enzyme systems that contributes to the increased oxidative status in the vasculature. FFA's also stimulate protein kinase C (PKC) that stimulates NADPH oxidase-2 (Nox2) to further produce O_2^- (Inoguchi et al., 2000). Similarly in diabetes, this chain of O_2^- producing reactions can lead to changes in the vasculature, e.g. uncoupling of eNOS, that can lead to the development of cardiovascular complications such as impaired vascular relaxation.

Chronic expansion of adipocytes occurring during obesity can lead to hypoxia and inflammation of WAT (Després and Lemieux, 2006). As a result of this WAT

inflammation causes the release of several cytokines such as (TNF-α), interleukin (IL)-6, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and adipokines (i.e. leptin and resistin) (Ouchi et al., 2011). This can lead to the onset of insulin resistance and in combination with obesity and low grade inflammation can lead to the eventual onset of type 2 diabetes (Lopategi et al., 2016). All of these physiological processes, and others which will be discussed later in this chapter, place obese patients at an increased risk of developing endothelial dysfunction and cardiovascular complications such as stroke and coronary artery disease (Caballero, 2005). The connection between diabetes, obesity and CVD is illustrated in Figure 1.10.

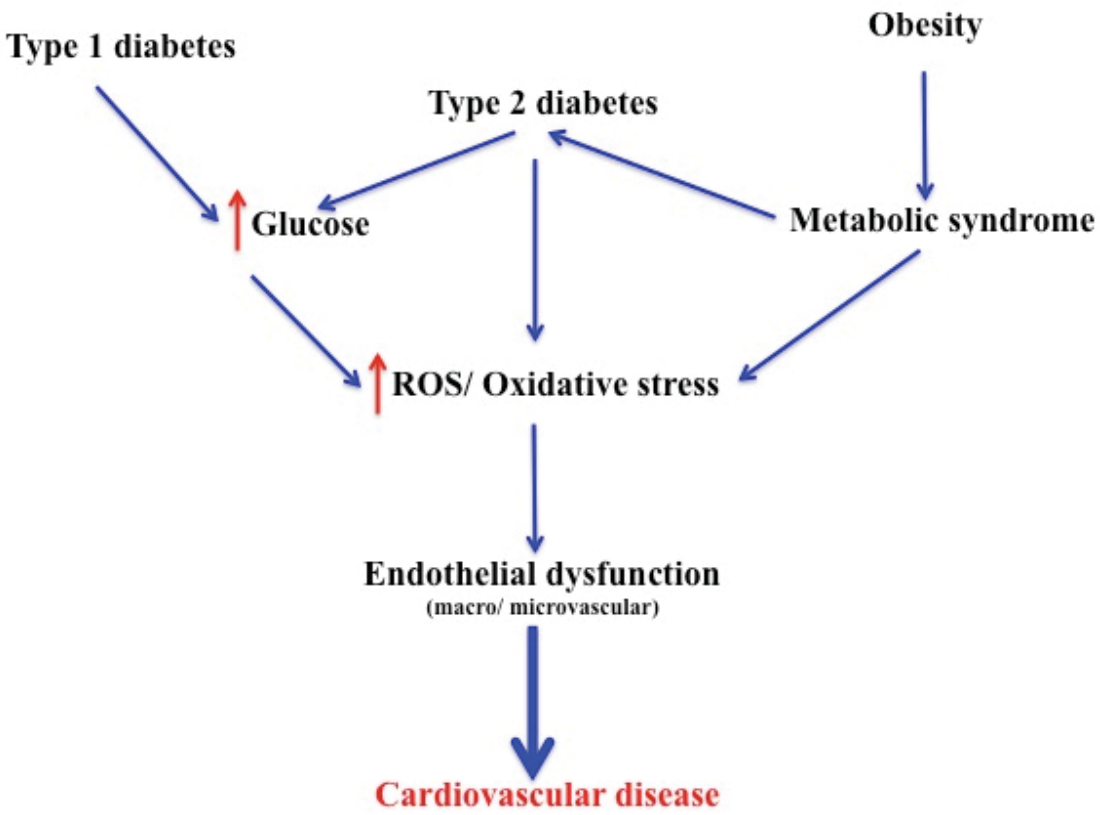


Figure 1.10 A schematic representation of the connection between obesity, diabetes and CVD: Mechanism and consequences of hyperglycaemia-induced oxidative stress: O_2^- production is increased via the up-regulation of several physiological pathways, a consequence the bioavailability of NO decreases and O_2^- production increases leading to the development of CVD.

1.6 Proposed mechanisms for oxidative stress during diabetes

Evidence of increased oxidative stress during diabetes was reported as early as 1979 by Sato et al. (1979) who reported increased levels of lipid peroxides in the plasma of type 2 diabetic patients. Since then various mechanisms have been proposed through which oxidative stress is increased during diabetes. These include mitochondria-induced oxidative stress, hyperglycaemia-induced oxidant stress, ROS associated oxidant stress, advanced glycation end product (AGE) formation, PKC activation and the hexosamine pathway.

1.6.1 Mitochondria-induced oxidative stress.

The mitochondria are the largest producers of O_2^- in humans under normal homeostatic conditions. ATP synthesis is driven by a proton gradient that is generated by protons entering the intermembrane space through protein complexes I, III and IV (Figure 1.11) (Giacco & Brownlee, 2010). This process is known as the electron transport chain. During diabetes, due to increased glucose concentrations, excessive NADH and $FADH_2$ enter the electron transport chain (Mehta et al., 2006). This leads to an increase in the proton gradient in the inner mitochondrial membrane at the level of complex III, which is the rate-limiting enzyme of oxidative phosphorylation. This causes electrons to back up at the level of complex III consequently generating more O_2^-. The O_2^- is then converted into cytotoxic H_2O_2. H_2O_2 can be converted into H_2O and O_2 by the actions of CAT and GPx (Figure 1.7) (Stephens et al., 2008). However, these enzyme systems can also be affected (e.g. decreased GPx activity) during diabetes due to the decreased bioavailability of cofactors such as NADPH (Figure 1.12). There is also evidence suggesting that mitochondria-induced oxidative stress occurring in diabetes

may activate other O_2^- generating pathways eg: the NADPH oxidases, which further exacerbates O_2^- production and oxidative stress (Giacco & Brownlee, 2010).

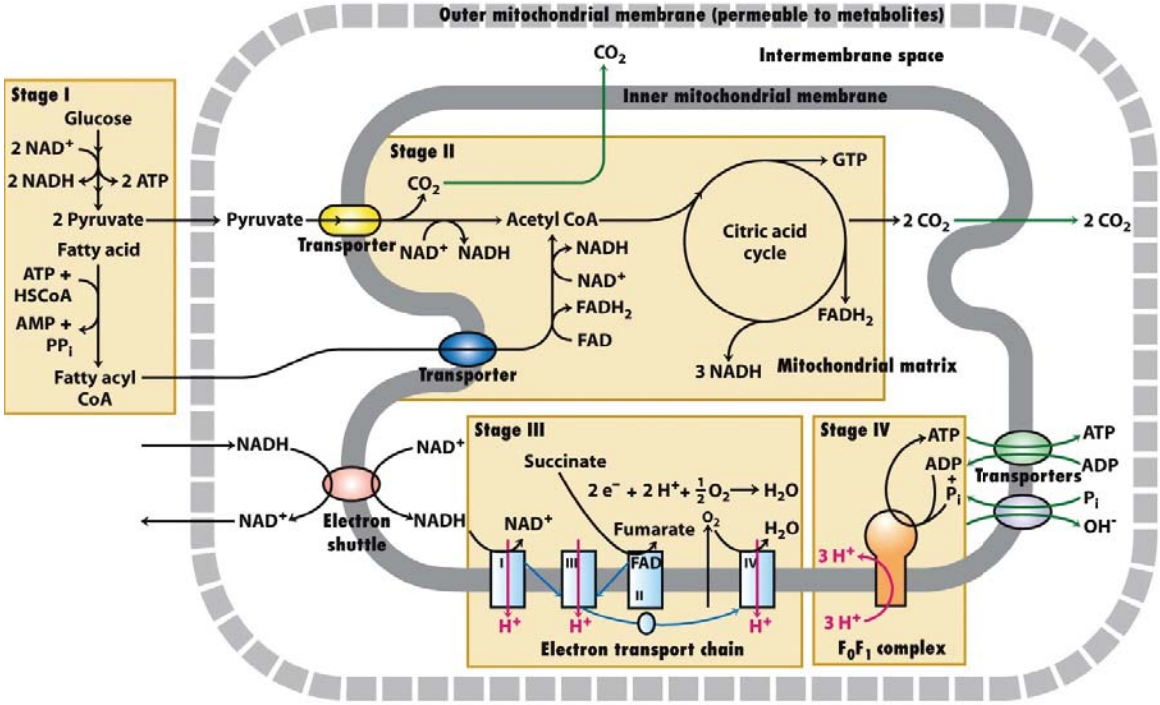


Figure 1.11 Glucose metabolism through the Krebs (TCA) cycle and electron transport chain (ETC): In the mitochondria during obesity oxidative stress may increase through increased $FADH_2$ and NADH influx in the electron transport chain increasing the proton gradient across the inner mitochondrial membrane leading to potential saturation of the system leading to uncoupling of the electron transport chain and oxidative phosphorylation. *Image taken from Lodish et al. (2007).*

1.6.2 Hyperglycaemia-induced oxidative stress and the polyol pathway.

One of the main mechanisms through which hyperglycaemia can increase oxidative stress and initiate cardiovascular complications in the vasculature is via the polyol pathway (Mehta et al., 2006). Aldose reductase is the enzyme responsible for reducing toxic aldehydes into inactive alcohols. During hyperglycaemia however, aldose reductase can also reduce glucose to sorbitol which is later oxidised to fructose (Figure 1.12) (Brownlee, 2005). This series of reactions is referred to as the polyol pathway. During this reaction, aldose reductase consumes NADPH, which is vital to the regeneration of reduced glutathione (GSH) (Figure 1.7). GSH is the molecule which is oxidised into oxidised glutathione (GSSG) by the actions of GPx which, as mentioned in the previous section, is one of the enzymes responsible for the neutralisation of H_2O_2 (Mehta et al., 2006). This depletion of NADPH and decreased bioavailability of GSH further exacerbates oxidative stress. The contribution of the polyol pathway during diabetes has been highlighted in many studies, for example Lee and Chung (1999) who demonstrated the role of increased oxidative stress in the development of diabetic cataract as a direct outcome of increased activation of the polyol pathway. Polyol pathway (Figure 1.12) activation and diabetes/ hyperglycaemia-induced complications is very common in organs that do not require insulin for the uptake of glucose such as blood vessels, retina and nerves and is a very common cause of endothelial dysfunction with diabetes related cardiovascular complications (De Vriese et al., 2000, Fortes et al., 1983). Oyama and colleagues (2006) demonstrated that in the presence of high glucose, endothelial cell damage may occur as a result of increased oxidative stress. They were further able to demonstrate that upon blocking the polyol pathway, oxidative stress was

decreased suggesting that the polyol pathway is a major source of oxidative stress in endothelial cells in the presence of high glucose/ diabetes (Oyama et al., 2006).

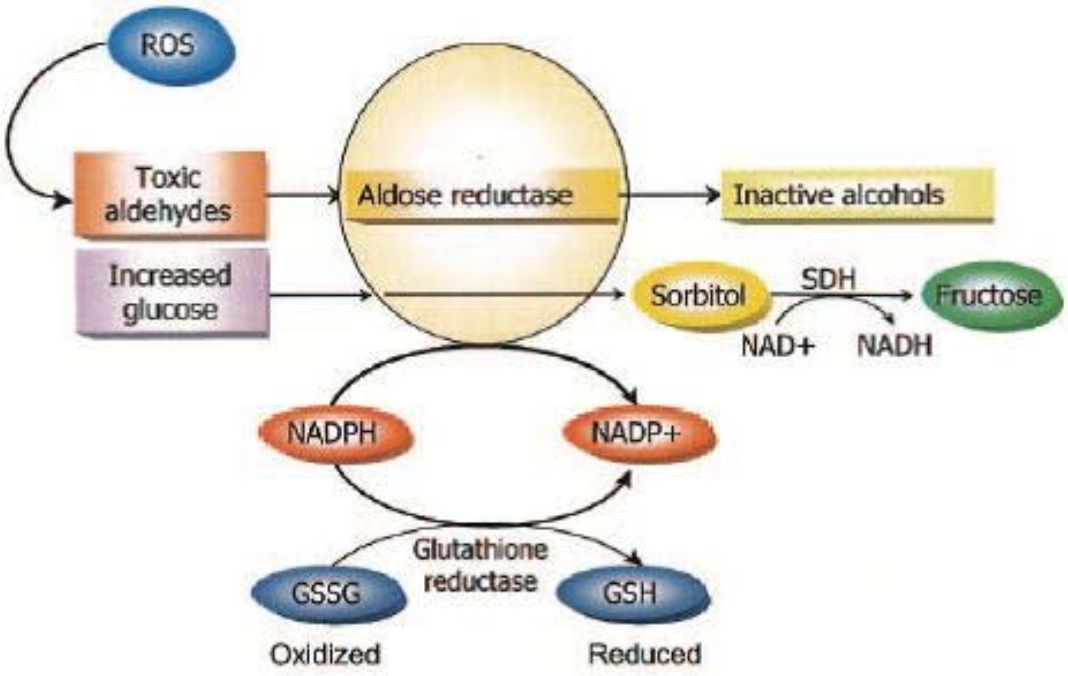


Figure 1.12 The polyol pathway: Hyperglycaemia increases influx of glucose into the polyol pathway which increases glucose oxidation to fructose. This consumes NADPH in the process which is necessary for GSH regeneration. *Taken from Brownlee (2005).*

1.6.3 Advanced glycation end product (AGE) formation.

Another mechanism through which hyperglycaemia can increase oxidative stress during diabetes is glucose mediated non-enzymatic glycosylation of proteins. This reaction (Maillard reaction) (Figure 1.13) leads to the formation of an unstable Schiff base which is characterized by covalent bonds between the amine group of the protein and the aldehyde group of the glucose molecule (Stephens et al., 2008). The Schiff base then undergoes further rearrangement and oxidation forming Amadori products (Maritim et al., 2003). Amadori products have a slow turnover rate (approximately 28

days), and undergo several dehydration reactions and rearrangements to form advanced glycation end products (AGEs) (Stephens et al., 2008, Maritim et al., 2003). Once formed, AGEs can bind to the AGE receptor; RAGE, the interaction between AGE and its receptor increases the production of $ONOO^-$ (Rojas and Morales, 2004). During diabetes there is increased production of AGE that can lead to increased vascular permeability, cell adhesion molecule expression and cell migration e.g. leukocytes (Rojas and Morales, 2004). All of these events are involved in the pathophysiology of CVD. Therefore increased AGE formation during diabetes may further compromise the ability of the vasculature to deal with oxidant stress and thus accelerate the development of CVD.

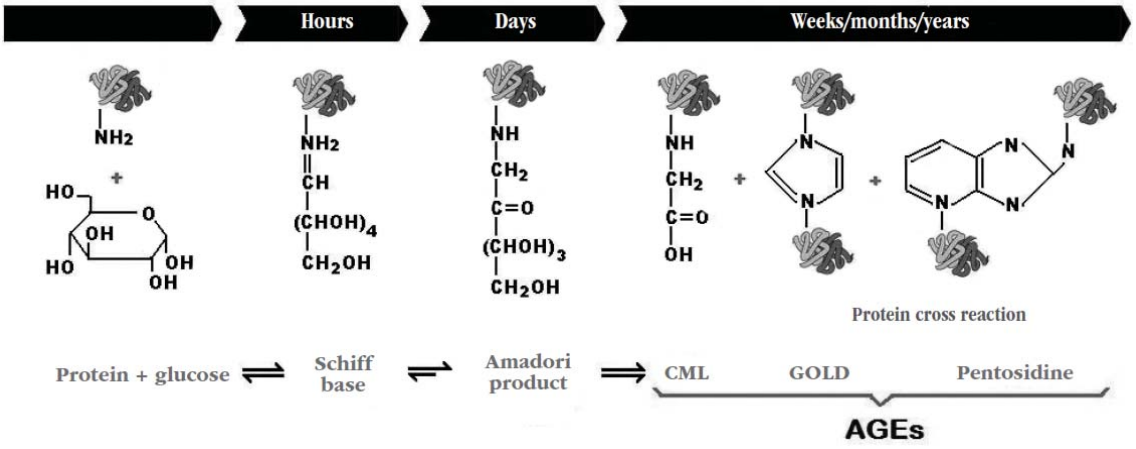


Figure 1.13 The formation of AGEs via the Maillard reaction: GOLD = glyoxal-lysine dimer, CML = Nε-(carboxymethyl)lysine, AGEs = advanced glycation end products. *Taken from Oliveira et al. (2013).*

1.6.4 PKC activation and NADPH oxidases.

Protein kinase C (PKC) is a family of serine/threonine enzymes which participate in a diverse range of cellular processes including; receptor desensitization, modulating

membrane structure, transcription regulation, mediating immune responses and cell growth regulation (Newton, 1995).

During diabetes, increased PKC activation may activate another family of enzymes known as Nox (Paravicini and Touyz, 2008). Nox are a family of enzymes that consists of seven isoforms however only four of them are expressed on endothelial cells (Nox1, 2, 4 and 5) (Drummond and Sobey, 2014). Nox (1, 2 and 5) produce O_2^- by participating in a one electron reduction of oxygen using NADPH as the electron source (Figure 1.14) (Sugamura and Keaney, 2011, Brandes et al., 2014).

Studies that have looked at the effect of PKC activation during diabetes include Inoguchi et al., (1992) who demonstrated increased PKC activation and diacylglycerol (DAG) concentrations in type 1 diabetic rat aorate and heart. Quagilaro et al., (2003) also demonstrated that exposure of human endothelial cells to high glucose increased PKC-induced activation of Nox leading to an increase in ROS production and cellular apoptosis. Nox related oxidative stress can also contribute to CVD related pathologies e.g. cardiac remodelling post myocardial infarction, ischaemia-induced angiogenesis and atherosclerosis (Judkins et al., 2009, Looi et al., 2008). Nox derived ROS during diabetes also activate a series of pathological cellular processes including initiating lipid peroxidation, stimulating kinases and stimulation of proinflammatory genes, impairment of vasorelaxation and most importantly, inactivation of NO (Paravicini and Touyz, 2008). Hence it has been proposed that increased PKC dependent Nox activation has severe pathological consequences where ROS production ultimately

becomes a self-generating process contributing to endothelial dysfunction and diabetes related complications

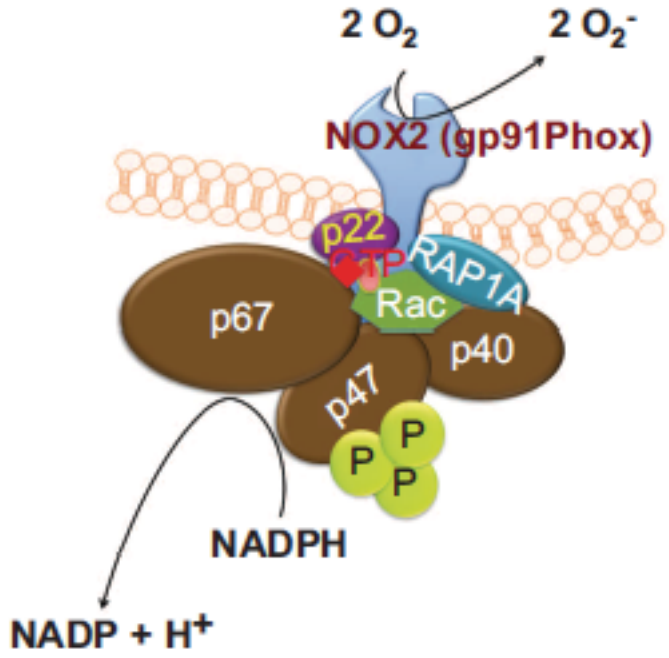


Figure 1.14: Nox2 structure: The Nox2 enzyme consists of two catalytic subunits; gp91-phox (Nox2), and p22-phox domains. Nox2 activation leads to translocation of the gp91 phox and Rac subunits that allows superoxide to be produced from oxygen being reduced by using NADPH as the source of the electron. Taken from (Panday et al., 2015).

1.6.5 Hexosamine pathway.

During diabetes (types 1 and 2) there is an increased reliance on fatty acid metabolism as a source of energy (Giacco and Brownlee, 2010). Energy in the body is derived from glycolysis from the conversion of glucose-6-phosphate into pyruvate. Fructose-6-phosphate is produced during one of the steps of glycolysis. It is also produced in the pentose phosphate pathway (PPP), a physiological process that produces NADPH and ribose-5-phosphate that is necessary for nucleic acid synthesis (Figure 1.15)

(Wamelink et al., 2008). During diabetes, fructose-6-phosphate is shunted from the glycolysis and PPP into the hexosamine pathway via the actions of the enzyme fructose-6-phosphate amidotransferase (GFAT) where it is then converted into UDP (uridine diphosphate)-*N*-acetylglucosamine (Figure 1.15) (Brownlee, 2001). This molecule is utilized by specific *O*-G1cNAc transferase enzymes for post-translational modification of the serine and threonine residues on the cytoplasmic and nuclear proteins (Giacco and Brownlee, 2010). This can then lead to activation of important genes such as TGF-α, TGF-β and plasminogen activator inhibitor (PAI)-1 through mechanisms which are unclear. These genes are responsible for vascular smooth muscle proliferation, cell differentiation and apoptosis that subsequently can impair vascular function and accelerate CVD related processes. Increased expression of these genes has been demonstrated during diabetes or in the presence of high glucose (Du et al., 2000, Chen et al., 1998). Inhibition of the GFAT enzyme has also been demonstrated to inhibit TGF-α transcription occurring as a result of hyperglycaemia (Kolm-Litty et al., 1998) indicating that inhibition of the hexosamine pathway can be beneficial to prevent the onset of diabetes related CVD complications. The hexosamine pathway will be further discussed in section 1.7.

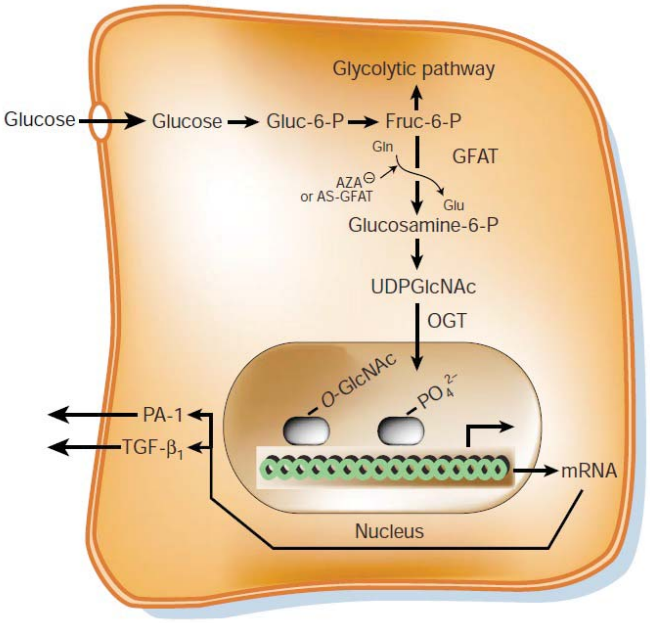


Figure 1.15: The hexosamine pathway: Fructose-6-phosphate (F6P) can be formed through glycolysis or from the pentose phosphate pathway. During diabetes F6P can be shunted from these pathwys into the hexosamine pathway. The hexosamine pathway can lead to activation of TGF-β via a series of reactions that can damage DNA. TGF-β is involved in the pathogenesis of CVD. *Taken from Brownlee (2001).*

1.7 Proposed mechanisms for oxidative stress during obesity

Increased oxidative stress during obesity is one of the major causes of endothelial dysfunction that eventually leads to obesity related cardiovascular complications such as atherosclerosis. There are several mechanisms and potential sources of ROS (Figure 1.16) that increase oxidative stress during obesity. These mechanisms include fatty acid oxidation, increased mitochondrial oxidative stress, hexosamine pathway and decreased vitamin E concentration.

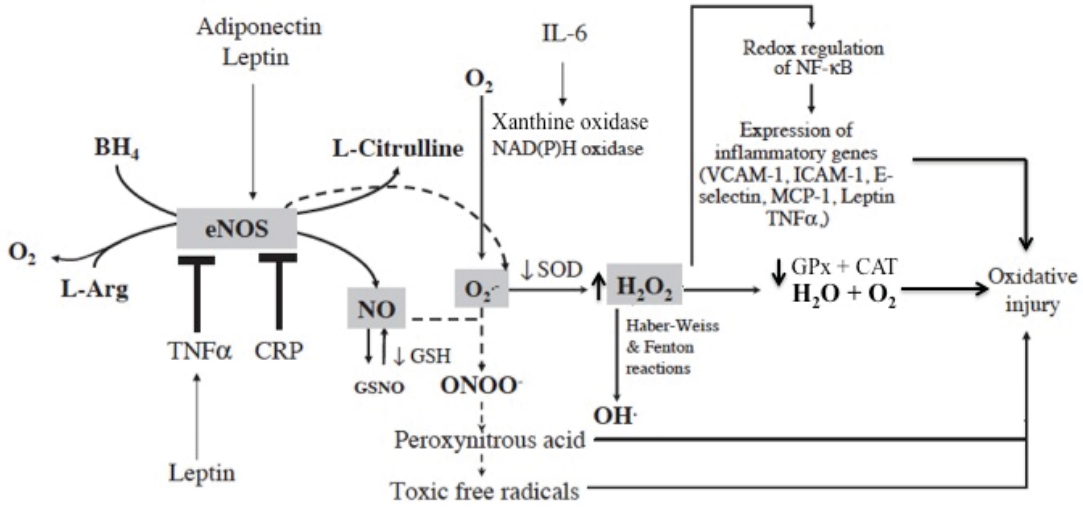


Figure 1.16: Potential sources of ROS during obesity: Obesity mostly occurs as a result of consuming more kilojoules than is expended. This can cause an increase in O_2^- production and formation of $ONOO^-$, a key mediator of lipid peroxidation from sources such as increased IL-6-induced xanthine oxidase activation during obesity. NO is produced from the conversion of L-Arginine into L-Citrulline and during obesity increased O_2^- can lead to eNOS uncoupling resulting in endothelial dysfunction. The formation of $ONOO^-$ decreases NO bioavailability and increases lipid peroxidation and foam cell formation in atherosclerotic lesions. Uncoupling of eNOS further increases oxidative stress and the formation of other ROS e.g. H_2O_2 that subsequently decrease the activity of GPx and CAT enzymes to further increase oxidative injury. Adapted from Avogarro and Vigili de Kretzenberg (2005).

1.7.1 Fatty acid oxidation and mitochondrial oxidative stress.

Obese people, even in the absence of diabetes, can also have increased oxidative stress due to increased FFA oxidation. When energy intake exceeds energy expenditure, there is an increase in pyruvate that is the substrate for the TCA cycle (Krebs cycle). Pyruvate is produced from glycolysis resulting from the breakdown of monosaccharides, fatty acids and amino acids (Figure 1.17). Pyruvate enters the inner mitochondrial membrane to enter the Krebs cycle. Pyruvate is converted into a pyruvate dehydrogenase complex (acetyl CoA) which undergoes a series of chemical reactions to produce $NADH^+$,

$FADH_2$ and ATP (Quijano et al., 2016) which are cofactors for many physiological reactions such as the pentose phosphate pathway (PPP) and eNOS derived NO production. The Krebs cycle also produces oxaloacetate and isocitrate which are the building blocks for many amino acids and other physiological molecules (Umbarger, 1978). The cofactors which are produced from the TCA cycle are also vital for ATP production via the processes of oxidative phosphorylation or the electron transport chain that occur in the inner mitochondrial membrane.

During obesity there is excessive energy substrate in the body which then undergoes glycolysis for ATP production. This process is anaerobic thus making it extremely energy inefficient (4 ATP molecules/ glucose molecule). The pyruvate then produced from glycolysis is shuttled into the mitochondria into the Krebs cycle and eventually into the inner mitochondria membrane. As mentioned previously in section 1.6.1 complex III in the inner mitochondrial membrane is the rate-limiting step of ATP production in the mitochondria. Therefore, due to excessive substrate availability during obesity, similarly to diabetes, there is an increase in the proton gradient in the inner mitochondrial membrane that eventually exceeds the capacity of oxidative phosphorylation to produce ATP further increasing oxidative stress. This is known as uncoupling of the ETC (Martin and McGee, 2014, Forbes and Cooper, 2013).

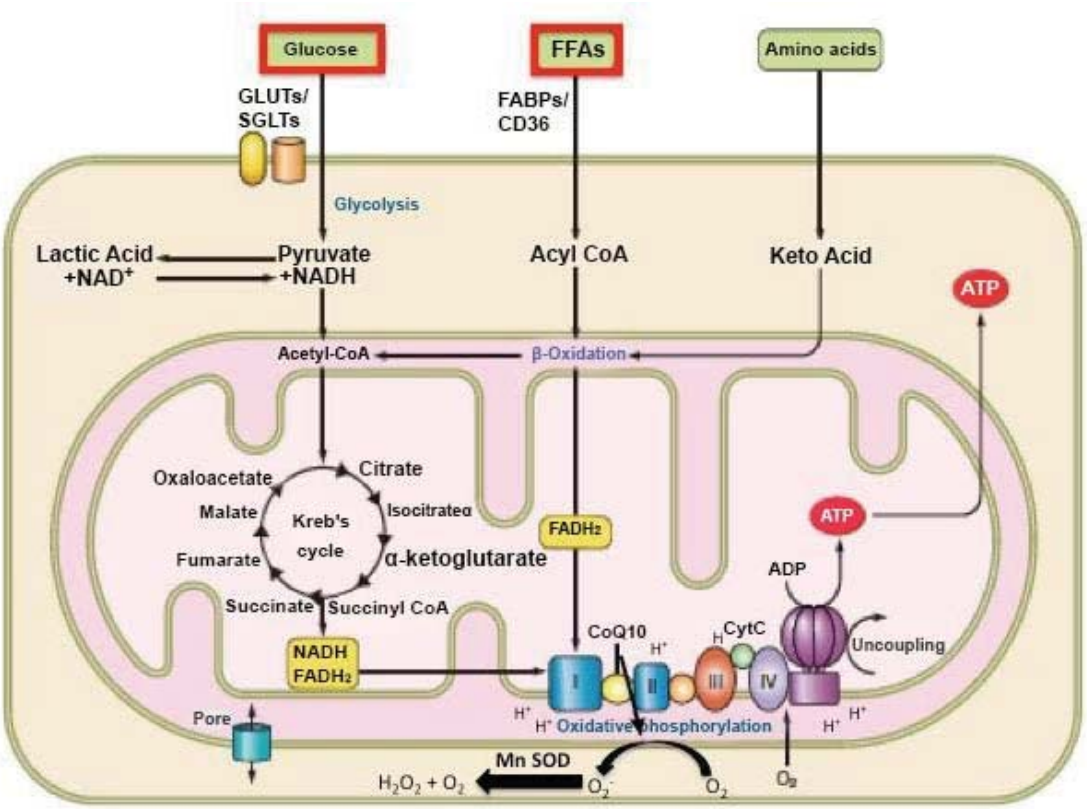
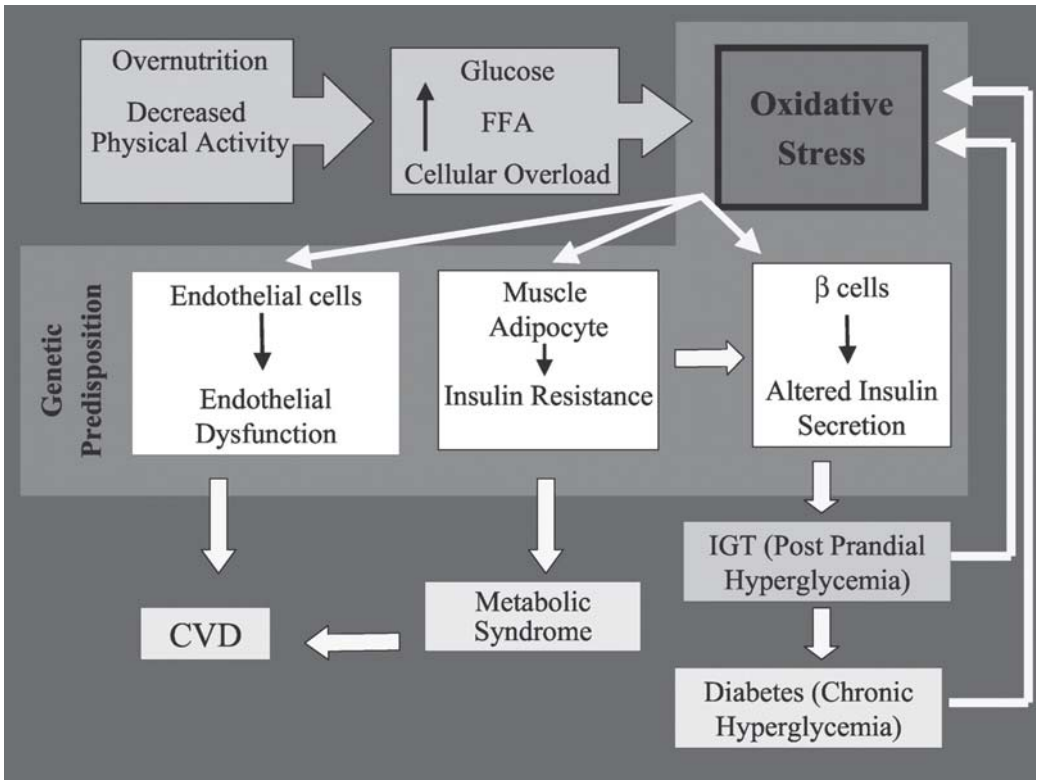


Figure 1.17 The tricarboxylic acid cycle, the ETC and oxidative phosphorylation: During obesity and the increased consumption of sugar and fat there is increased substrate entering the cell and entering the Krebs cycle. The $FADH_2$ produced enters the ETC and oxidative phosphorylation. This causes an increase in O_2^- from the mitochondria increasing oxidative stress. Also complex III is the rate limiting step in the ETC which eventually causes a build up of protons in the inner mitochondrial membrane causing the ETC and oxidative phosphorylation processes to uncouple subsequently further exaggerating oxidative stress. *Adapted from Forbes and Cooper (2013).*

1.7.2 ROS production, adipose tissue and insulin resistance

Under normal physiological conditions, FFAs are an important energy source for most body tissues and are the primary oxidative fuel for the liver, resting skeletal muscle, the renal cortex and the myocardium. Free fatty acids are a particularly important energy source during starvation, exercise and pregnancy, as they allow the preservation of

glucose for cerebral requirements (Boden and Shulman, 2002). However, overproduction of FFAs, which occurs during obesity, can cause a number of pathological processes, one of which is insulin resistance, a hallmark characteristic of metabolic syndrome.



IGT: Impaired glucose tolerance CVD: Cardiovascular disease

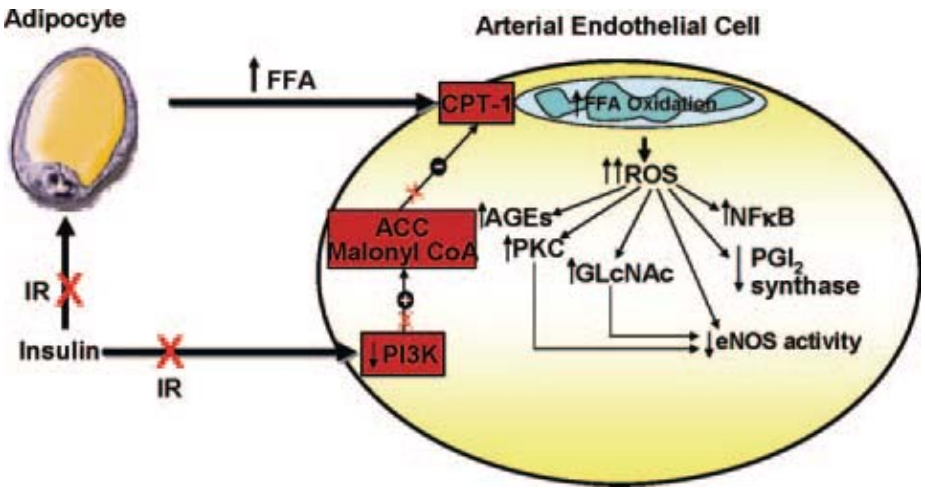
Figure 1.18 The relationship between obesity, increased FFA/ free glucose and insulin resistance, diabetes and cardiovascular disease: Overnutrition and decreased physical activity leads to increased glucose and FFA's entering adipocytes. Insulin is immediately released to allow glucose uptake however the excessive insulin release leads to insulin resistance. Insulin resistance coupled with oxidative stress in turn induces endothelial dysfunction. Endothelial dysfunction may lead to the development of CVD related pathologies. *Taken from Ceriello and Motz (2004).*

As mentioned in the previous section FFAs are a major source of ROS in obesity. Amongst the many molecules secreted by adipose tissue, adiponectin is a cytokine highly abundant in plasma and only expressed in adipose tissue (Yadav et al., 2012). Adiponectins have an anti-inflammatory effect on endothelial cells to improve vascular function (Hotta et al., 2000). Adiponectin concentration is inversely proportional to fat content (Lopez-Jaramillo, 2016). Saito et al. (2008) have previously demonstrated increased oxidative stress in the plasma and visceral adipose tissue of obese patients. These patients also had low levels of adiponectin that was increased by treatment with HMGCoA reductase inhibitor pravastatin (cholesterol lowering statin). They also demonstrated decreased oxidative stress with pravastatin treatment. Obesity has been demonstrated to decrease adiponectin levels. Conversely, increased adiponectin has been demonstrated to decrease circulating FFAs and increase insulin sensitivity (Lihn et al., 2005). Insulin resistance is a major predecessor of CVD (Figure 1.18).

Insulin is the major blood glucose-regulating hormone of the body and functions to promote glucose uptake from the blood into body organs. Insulin is produced in the islet β-cells of the pancreas and is very sensitive to oxidative stress. This is due to a lack of endogenous antioxidants in pancreatic- β cells i.e. SOD, GPx and CAT (Ceriello and Motz, 2004, Tiedge et al., 1997).

Insulin resistance is the inability of the body to respond to endogenous insulin resulting in hyperglycaemia and can progress into type 2 diabetes (Arner, 2002). During insulin resistance due to decreased insulin-mediated glucose uptake can lead to increased oxidative stress (Figure 1.19). There have been studies that have demonstrated increased oxidative stress in skeletal muscle leading to decreased glucose uptake by skeletal muscle (Roy et al., 1998). Skeletal muscle is the major consumer of insulin-

mediated glucose uptake. Excessive glucose in skeletal muscle is known to cause glucose toxicity whereby muscles can decrease glucose uptake by decreasing GLUT4 expression, which is the protein involved in insulin-mediated glucose uptake (Kim et al., 2001). Therefore it can be suggested that oxidative stress leads to decreased glucose uptake that can subsequently contribute to hyperglycaemia and insulin resistance (Figure 1.17). It may also be suggested that during obesity, as a result of increased caloric intake, insulin resistance can be a protective mechanism whereby muscles protect themselves from glucose toxicity (Unger, 2003). This was demonstrated by Hoehn et al. (2009) that demonstrated that insulin resistance is a defence mechanism in adipocytes, myotubes and mice.



IR: Insulin Resistance NFκB:

PI3K: PGI_2 synthase:

Figure 1.19 The effect of FFAs and insulin resistance on endothelial oxidative stress eNOS activity: An increase in adipose tissue mass and FFA's in the vasculature leads to increased FFA oxidation that subsequently increases ROS production this has a downstream affect on AGE and PKC production that reduces eNOS activity and consequently NO production. *Taken from Giacco and Brownlee (2010).*

1.7.3 Glycolysis, ROS production and the hexosamine pathway.

The consumption of excess calories (nutrient excess) has deleterious effects on glycolytic pathways of the body. The hexosamine pathway can increase oxidative stress in both diabetic and obese subjects. The hexosamine pathway accounts for approximately 3% of total glucose utilized by the body (Buse et al., 2007) however during obesity its activity can be significantly upregulated. The substrate for the hexosamine pathway is fructose-6-phosphate that is a produced in glycolysis and pentose phosphate pathway (PPP). The PPP is the physiological pathway that provides substrate for the TCA cycle and the electron transport chain/ oxidative phosphorylation. The hexosamine pathway is responsible for the synthesis of the glycosyl side chain of glycolipids, proteoglycans and glycoproteins (Buse, 2005).

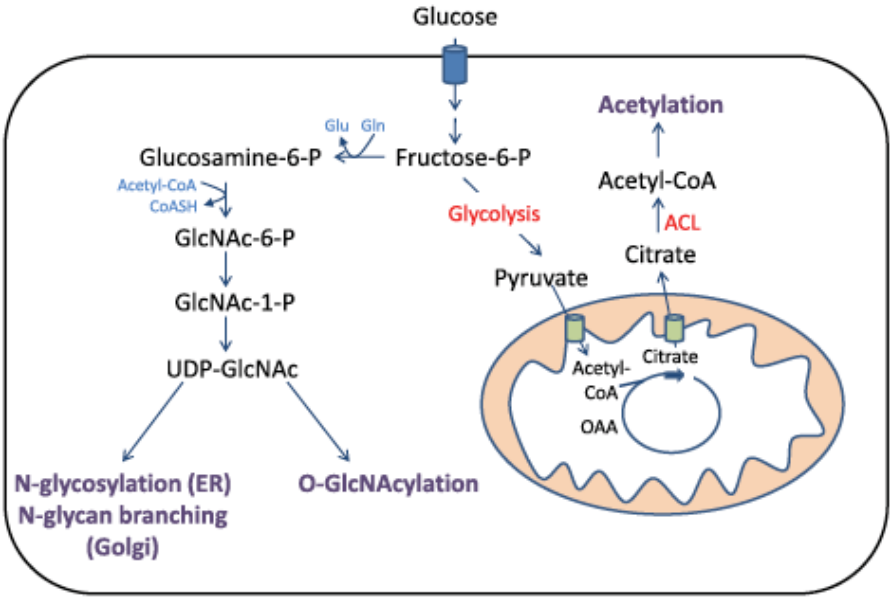


Figure 1.20 The hexosamine pathway: During obesity there is an increased influx of substrate (glucose (glucose-6-phosphate; G6P)) entering the glycolysis pathway, however there is also an increased influx of substrate entering the hexosamine pathway that concerts glucose into glucosamine-6-phosphate. Following several reactions UDP-GlcNAc is produced that is necessary for the glycolipid synthesis. Sustained long-term UDP-GlcNAc production that occurs during obesity is shown to inhibit eNOS activity and is involved in the pathophysiology of insulin resistance. *Taken from Wellen and Thompson (2010)*.

During obesity or nutrient excess, when there are increased glucose and FFAs circulating in the blood, there is an increased level of fructose-6-phosphate that is shunted from glycolysis and the PPP into the hexosamine pathway. The end-product from the hexosamine pathway is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (Figure 1.20) which is necessary for nucleocytoplasmic *O*-GlcNAc protein modification and N-linked glycosylation in the Golgi and endoplasmic reticulum (Wellen and Thompson, 2010, Love and Hanover, 2005). *O*-GlcNAcylation is of particular importance, especially to aortic smooth muscle cells, where increased *O*-GlcNAcylation has shown to inhibit the Akt activation site of eNOS (Musicki et al., 2005). This subsequently decreases eNOS activity and impairs vascular relaxation contributing to the pathogenesis of obesity- and diabetes -induced cardiovascular disease (Giacco and Brownlee, 2010).

One of the major enzymes involved in the hexosamine pathway is glucosamine—fructose-6-phosphate aminotransferase (GFAT). Over expression of GFAT has been demonstrated to be destructive to pancreatic-β cells and increase the oxidative status in β-pancreatic cells and eventually lead to diabetes (Robertson, 2004). One study has also demonstrated that hyperglycaemia can suppress insulin gene transcription and decrease insulin-mediated glucose uptake (Robertson et al., 1992). Therefore suggesting that up regulation of the hexosamine pathway occurs during obesity as a mechanism to decrease mitochondrial-induced oxidative stress to reduce substrate entering the TCA cycle and subsequently the ETC. However, hexosamine up-regulation leads to pancreatic-β cell destruction that leads to increased oxidative stress in the pancreas and insulin deficiency (Prentki and Nolan, 2006).

1.7.4 Decreased vitamin E and obesity.

Vitamin E (Figure 1.20), is a lipid soluble vitamin that acts as a chain breaking antioxidant that scavenges peroxyl radicals to prevent lipid peroxidation in plasma membranes (Traber and Stevens, 2011). The most widely studied form of vitamin E is α-tocopherol. Vitamin E levels have been reported to be significantly lower in obese patients in comparison to healthy people (Traber, 2014). A study conducted by Strauss (1999) demonstrated that obese children had significantly lower levels of fat-soluble vitamins (α-tocopherol and β-carotene) in comparison to healthy weight children. Interestingly, a recent study published by Mah et al. (2015) has also found that despite adequate α-tocopherol (vitamin E) intake, obese patients with metabolic syndrome had significantly lower levels of plasma α-tocopherol. The study also demonstrated decreased absorption of α-tocopherol in obese metabolic syndrome patients in comparison to healthy subjects. This was attributed to increased basal inflammation and increased oxidative stress that is experienced during metabolic syndrome.

1.7.5 Antioxidants and endothelial function.

As discussed in sections 1.6 and 1.7, oxidative stress is a major cause of endothelial dysfunction. Vascular endothelium is highly enriched with exogenous (vitamins A&E) and endogenous (SOD, GPx and CAT) antioxidant systems that allow it to serve as an antioxidant barrier hence protecting the vasculature from oxidative damage (Lazo-de-la-Vega-Monroy and Fernndez-Mej, 2013). However, during diabetes and obesity, due to the increased production of ROS and a significant reduction in the antioxidant capacity of endogenous antioxidant systems, antioxidants may serve as a useful therapy to alleviate oxidative stress and thus prevent vascular complications such as CVD.

There has been a spectrum of antioxidants that have been used to improve endothelium-dependent relaxation in animal studies. Leo and colleagues (2011) demonstrated that 3',4'-dihydroxyflavonol was able to improve endothelium-dependent relaxation in mesenteric arteries from type 1 diabetic rats after one week of treatment. They also demonstrated that the antioxidant was able to reduce oxidant stress and prevent eNOS uncoupling indicating that eNOS is an important contributor in the maintenance of endothelium-dependent relaxation (Leo et al., 2011a). Other antioxidants that may improve endothelial function during diabetes include angiotensin converting enzyme (ACE) inhibitors, which have been reported to improve endothelial function in the right common femoral artery of type 1 diabetic patients (Arcaro et al., 1999). The SOD mimetic tempol has also been demonstrated to improve endothelium-relaxation in the STZ rat (Nassara et al., 2002). In addition, polyphenolic tea compounds have been demonstrated to reduce oxidative stress in the blood in an animal model of insulin resistance (Hininger-Favier et al., 2009).

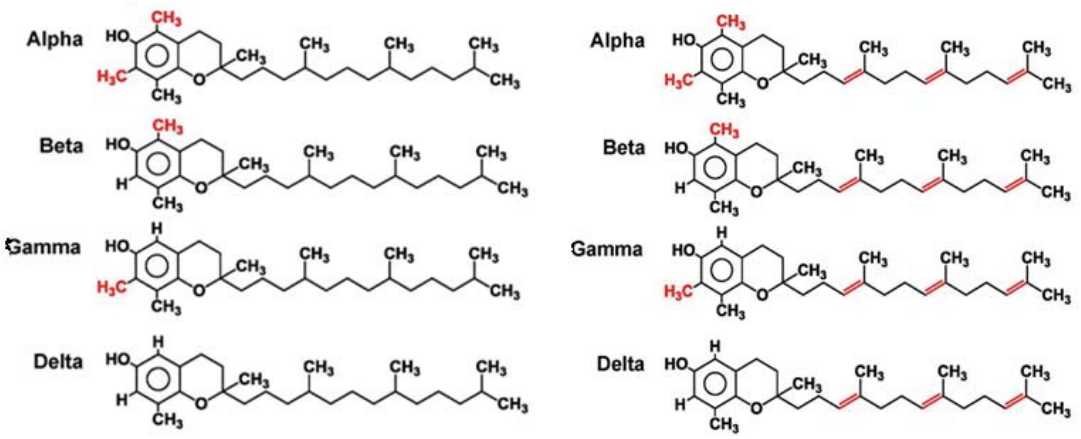
When translating the animal research outcomes into large scale human clinical trials, most antioxidants and their ability to reduce cardiovascular pathologies has produced disappointing results (Leopold, 2015). This includes large scale clinical trials using antioxidants such as selenium where long term supplementation was not able to reduce cardiovascular mortality or reduce the risk of cardiovascular events (Rees et al., 2013). Ascorbic acid (vitamin C) and its effect on endothelial function during CVD has also been extensively studied with mixed outcomes. For example, a study conducted by Antoniades et al. (2004) studied the effect of vitamin C on endothelial function in patients with coronary artery disease (CAD) and diabetes. They demonstrated that 4-week vitamin C supplementation significantly improved forearm vasodilatation in

patients with CAD plus diabetes however, they were not able to demonstrate the same effect in diabetic patients. Similarly a study conducted by Chen et al. (2006) was not able to demonstrate any significant effect of vitamin C supplementation in type 2 diabetic patients. Studies that have been able to demonstrate any significant benefit of vitamin C supplementation during CVD have usually had patients on extremely high concentrations of vitamin C (>500mg/day) (Ashor et al., 2014). Although this may improve cardiovascular outcomes, extremely high supplementation of vitamin C has shown to increase oxidative damage to peripheral blood lymphocytes in terms of modified DNA bases (Podmore et al., 1998).

Therefore, if increased oxidative stress is a major cause of endothelial dysfunction and the cardiovascular complications of diabetes and obesity then antioxidant therapy may alleviate or delay any subsequent cardiovascular complications.

1.8 Vitamin E

Vitamin E is a family of antioxidant molecules which consist of tocopherols and tocotrienols (Atkinson et al., 2008). Both tocopherols and tocotrienols have four chemically distinct isomers named alpha (α), beta (β), delta (δ) and gamma (γ) based on the positioning of the methyl molecules on the chromanol ring (Figure 1.20). Both tocopherols and toctrienols share structural similarities but they differ chemically in their chains as tocopherols have a phytyl chain and tocotrienols have an isoprenoid chain (Figure 1.21) (Aggarwal et al., 2010).



tocopherol (TC) **tocotrienol (T3)**

Figure 1.21 Chemical structure of tocopherols and tocotrienols: Tocopherols and tocotrienols are members of the vitamin E family that have 4 known isomers each. Both tocopherol and tocotrienols have a chromonal ring with and a hydroxyl group attached to the ring. The α TC/T3 has 3 methyl groups on the chromonal ring, δ -TC/T3 has 1 methyl group and β γ-TC/T3 have 2 methyl groups. The designation of the individual isomers is based on the number and position of the methyl groups on the chromonal ring (red).

Most of the scientific literature on vitamin E and its antioxidant properties has focused on tocopherols, especially *α*-tocopherol. This can be attributed to the relatively greater abundance of tocopherols in food sources in comparison to tocotrienols and the ease of extraction from natural sources, hence making it an extensively researched molecule.

1.8.1 Pharmacology of vitamin E.

The main function of vitamin E is to act as a chain breaking antioxidant and scavenge lipid peroxyls and O_2^- to prevent oxidative damage to membranes and DNA in biological tissues (Yoshida et al., 2003). Once vitamin E (e.g. *α*-tocopherol) scavenges lipid peroxyls or O_2^- it becomes a tocopheroxyl radical (Figure 1.21) (Kamal-Eldin and

Appelqvist, 1996). The tocopheroxyl radical is then recycled back to vitamin E by using vitamin C as an electron acceptor. The basic antioxidant action of vitamin E and how it is recycled is illustrated in Figure 1.22.

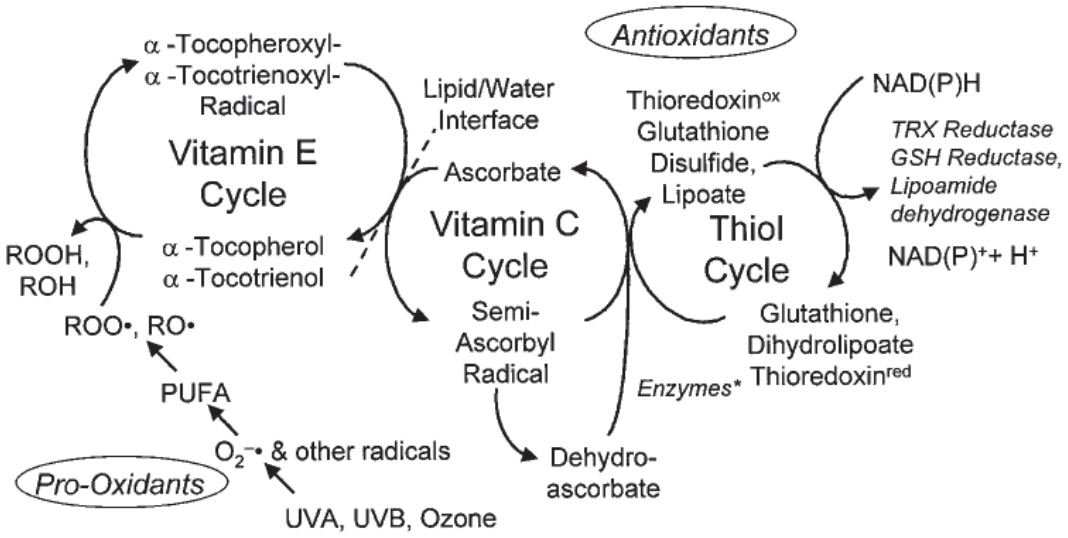


Figure 1.22 Vitamin E function: Vitamin E scavenges lipid peroxyls and is recycled back into vitamin E by the actions of vitamin C. This antioxidant system also requires GSH and NADPH to allow effective recycling of vitamin E. During states of increased oxidative stress may decrease the bioavailability of cofactors GSH and NADPH leading to decreased vitamin E recycling consequently affecting other enzyme system such as eNOS. *Taken from Packer et al., (2001).*

Vitamin E is found in the plasma membranes (lipid bilayer) of cells and is part of larger antioxidant network system rather than an antioxidant functioning in isolation (Atkinson et al., 2008). It is also the most abundant lipid soluble antioxidant found in membranes (Rigotti, 2007) that allows it to perform its antioxidant function effectively. Although all forms of tocopherols and tocotrienols are found in foods, the body only maintains α-tocopherol in the body it is one of the most important dietary antioxidants and all dietary guidelines of vitamin E intake are based on the consumption of α-tocopherol (Packer et al., 2001).

Vitamin E is solubilized into micelles by the actions of bile salts and intestinal lipids after which they are absorbed into the intestinal epithelium (Figure 1.23) (Traber et al., 1990). They are then actively transported into the bloodstream through various carriers including chylomicrons and mainly high and low density lipoproteins (Anwar et al., 2006, Perugini et al., 2000). They are then incorporated into membranes by the actions of the α-tocopherol transport protein (α-TTP) (Figure 1.22) (Manor and Morley, 2007).

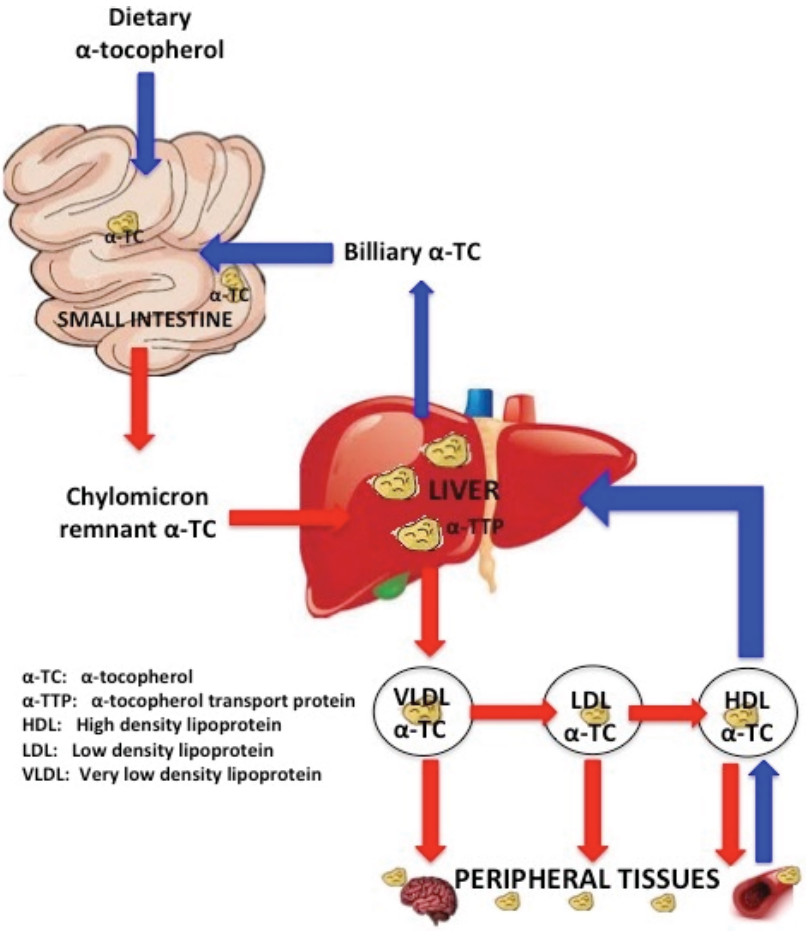


Figure 1.23 Vitamin E absorption and transport: α-TC is absorbed in the small intestine and incorporated into chylomicrons. They are then converted into remnant particles. Remnant chylomicron associated α-TC is transported to the liver and is selectively incorporated into VLDL's by the α-TTP and is re-secreted into circulation. Endogenous apoB–containing VLDL's and LDL's can exchange α-TC with HDL's where LDL and HDL are the main sources for the cellular uptake of α-TC in peripheral tissues. HDL's can also remove excess α-TC from cells. *Adapted from Mardones and Rigotti (2003)* .

The plasma concentration of vitamin E (α-tocopherol) in humans is reported to range from 15-20 μmol/L (Grzelińska et al., 2009, Schweigert et al., 2003) however this is not indicative of the concentration of vitamin E in cell membranes, the term "tocol sufficiency" which refers to the amount of vitamin E expressed as a mole percentage of the phospholipids, is a better indicator of vitamin E in a cell (Atkinson et al., 2008). However there is a lack of literature that measures vitamin E concentration in this manner and is variable from tissue to tissue (McMurchie and McIntosh, 1986). For example the concentration of vitamin E in human platelets is 0.1-0.15 mol% (Steiner, 1978) and 0.05-0.13 mol% in rat liver mitochondria (Gruger and Tappel, 1971). As mentioned previously, despite sharing structural similarity both tocopherols and tocotrienols differ functionally that gives each type of vitamin E unique properties which will be discussed further.

1.8.2 Tocopherols.

Tocopherols were the first identified components of vitamin E and are the most abundant. Sources of tocopherols include; various nuts (including, almonds, hazelnuts and pistachios), flaxseed, poppy seed and sunflower seeds (Aggarwal et al., 2010).

There have been numerous animal studies that have looked at the effect of vitamin E (α-tocopherol) and its ability to improve endothelial function in diabetic animal models. This includes a study by Keegan and colleagues (1995) who demonstrated an improvement in endothelium-dependent relaxation in type 1 diabetic rat aorta with a 1% dietary supplement of α-tocopherol. α-Tocopherol was also able to increase superoxide scavenging capacity in this study. This suggests that vitamin E exerts its

protective effects through its antioxidant activity. Another study conducted by Wigg and colleagues (2004) was able to demonstrate that α-tocopherol supplementation (1g/kg/day) improved endothelium-dependent relaxation in type 1 diabetic rat mesenteric arteries, which was attributed to an increased NO bioavailability. Also a reduction in vessel wall stress was observed as a decrease in the wall thickness of messenteric and femoral arteries (Wigg et al., 2004). This was potentially through vitamin E decreasing arterial wall thickness by directly affecting re-arrangement of the components and/or structure of the arterial walls. Therefore these and other studies indicate a potential for α-tocopherol as an effective antioxidant in alleviating vascular oxidant stress.

There have been several reports that α-tocopherol improves vascular function or reduces oxidant stress during diabetes in animals (Gazis et al., 1999, Karasu et al., 1997, Rosen et al., 1985) and small human trials (Skyrme-Jones et al., 2000, Bursell et al., 1999) large scale clinical trials have also conducted but with very disappointing results. This includes the HOPE study that was unable to demonstrate any long term benefit of vitamin E supplementation in preventing major cardiovascular events in patients with diabetes or vascular disease (Lonn et al., 2005). The HOPE study examined the effect of vitamin E (α-tocopherol) supplementation (400 IU/day) in patients with CAD for an average period of 4.5 years. Their findings revealed that if anything, vitamin E supplementation increased the incidence of heart failure in the supplemented patients. Another example was the Framingham study that demonstrated that vitamin E supplementation (100-600 IU/day, 67% participants had > 300 IU/day) did not reduce CVD related mortality or decrease the risk of CVD in patients with diabetes or pre-existing vascular disease (Dietrich et al., 2009). The alpha-tocopherol and beta-carotene

study (ATBC) (Leppälä et al., 2000) demonstrated no significant benefit of supplementation of 50mg/day of α-tocopherol on incident of myocardial infarction or fatal CAD. On the contrary, the study demonstrated an increased rate of fatal hemorrhagic stroke in the study's participants. Another large-scale clinical trial includes the Physicians Health Study II (PHS-II) (Gaziano et al., 2009). The PHS II study also did not demonstrate any long-term benefit of vitamin E or C supplementation on reducing the risk of prostrate or any cancer.

There have been many reasons put forward to explain why tocopherol supplementation studies (including the ones mentioned previously) have not proven successful in humans. Some suggestions include the advanced age of participants, the diabetes and/or vascular disease being too far advanced that treatment with antioxidants such as vitamin E is not beneficial, and the potential for tocopherols to become a pro-oxidant at high concentrations (Paravicini and Touyz, 2008, Bowry et al., 1992). This has led investigators to test different antioxidants that may be able to serve as a more useful antioxidant in ameliorating diabetes and obesity-induced endothelial dysfunction and its subsequent cardiovascular complications.

1.8.3 Tocotrienols

Tocotrienols, are a more recently identified but perhaps a more potent member of the vitamin E family (Serbinova et al., 1991). Tocotrienols were first described in 1964 when they were isolated from the latex of the rubber plant, *Havea brasiliensis* (Dunphy et al., 1965). Since then other richer sources of tocotrienols have been identified including palm oil, rice bran, wheat germ and barley (Aggarwal et al., 2010). Tocotrienols just like tocopherols have 4 chemical isomers and their nomenclature is

based on the position of the methyl molecules on the chromanol ring, identical to the nomenclature of tocopherols (Figure. 1.19).

Tocotrienols have a very similar chemical structure to tocopherols but are significantly more potent antioxidants than tocopherols. This was demonstrated by a study conducted by Serbinova and colleagues in 1991, revealing α-tocotreinol to be up to 50 times more potent than α-tocopherol as an antioxidant against (Fe^{2+} + ascorbate) and (Fe^{2+} + NADPH)-induced lipid peroxidation in rat liver microsomal membranes and 6.5 times more effective than α-tocopherol in reducing oxidative. The high potency of tocotrienols can potentially be attributed to the presence of double bonds in the phytyl chain making them highly lipid soluble and can therefore achieve high intracellular concentrations (Aggarwal et al., 2010).

Tocotrienols are also considered to possess other properties that tocopherols do not possess including anticholesterol (Pearce et al., 1992) and antithrombotic properties (Baliarsingh et al., 2005). Hence it could be hypothesised that tocotrienols may improve diabetes and obesity-induced endothelial dysfunction consequently preventing or delaying the onset of diabetes and obesity related complications such as CVD.

Studies investigating the effect of tocotrienols on vascular function include by Muharis et al. (2010). They demonstrated that acute exposure to tocotrienol rich palm oil fractions and α-tocopherol (1/mg/mL) significantly improved endothelium-dependent relaxation in type 1 diabetic and spontaneously hypertensive rats. However, whether tocotrienol rich palm oil is a more potent antioxidant than α-tocopherol was not studied as the study used the same dosage for both tocotrienols and α-tocopherol. The

antioxidant properties of tocotrienols was also reflected in their results that studied superoxide-scavenging capacity, which showed that α-tocopherol and their tocotrienol rich fraction possessed similar superoxide scavenging capacity at the same concentrations (0.3mg/mL-1mg/mL). Another study that has demonstrated tocotrienol rich fraction reducing vascular oxidant stress during diabetes is by Budin et al. (2009). They demonstrated that eight-week supplementation of the diet of type 1 diabetic rats with a tocotrienol rich fraction (200mg/kg/day) lowered blood glucose levels, decreased oxidant stress and improved the morphology of the aorta which was seen as a decrease in vascular smooth muscle cell proliferation, inhibition of dense amorphous material formation, more regular elastic lamina and a smooth tunica intima (Budin et al., 2009). Both of these studies involved extracts of palm oil that included some tocopherol as well as a mixture of tocotrienols. There appear to be no studies examining the antioxidant actions of specific tocotrienol isoforms in the vasculature. Thus there are studies with promising but limited data on the actions of mixed tocotrienols.

1.8.4 Tocomin.

Tocomin is a mixture that is derived from a palm oil extract rich in tocotrienols (tocotrienol rich fraction: 40%, and palm olein: 38%) but also containing some α-tocopherol (11%). It is manufactured by Carotech and is the drug of interest that will be studied in this thesis. The ability of tocomin to reduce oxidative stress has not been widely studied. A study conducted by Sen et al. (2000) demonstrated that tocomin supplementation in healthy women achieved plasma levels of tocotrienols 12 to 30 times more than the concentration of α-tocotrienol required to protect against stroke-related neurodegeneration. Another study conducted by the same research group

Khanna et al. (2005) later demonstrated that 8-week tocomin supplementation (1g/kg) protected spontaneously neuronal cells against glutamate- and stroke-induced neuro-degeneration.

The effect of tocotrienol rich fractions of palm oil on endothelial function during disease states where there is increased oxidative stress in the vasculature has not been extensively studied. A study conducted by Muharis et al. (2010) demonstrated that a palm oil fraction rich in tocotrienols possessed potent antioxidant capacity and improves endothelium-dependent relaxation in the arteries of STZ-induced type 1 diabetic rats and spontaneous hypertensive rats.

Hence based on the lack of current literature on the antioxidant activity and affect of tocotrienols on vascular function during disease states of increased oxidative stress such as diabetes and obesity, further investigation into the function of tocotrienols is warranted.

1.9 Hypothesis and Aims

Therefore the aims and hypotheses to be investigated were;

Aim 1: Compare vascular and antioxidant activity of three tocotrienol isomers (*α, δ* and *γ*) and tocomin to *α*-tocopherol. *β*-tocotrienol was not studied in this thesis because we were not able to obtain any *β*-tocotrienol. Also there has been no literature that has been able to demonstrate any pharmacological benefit of *β*-tocotrienol in any pathology (Ahsan et al., 2014).

Hypothesis 1: Tocotrienols are significantly more potent antioxidants compared to *α*-tocopherol.

Aim 2: Determine whether *α, δ* and *γ*-tocotrienols in isolation or in combination and tocotrienol rich tocomin can improve endothelium-dependent relaxation in the presence of oxidative stress.

Hypothesis 2: Tocotrienols and tocomin improve endothelium-dependent relaxation in the presence of oxidative stress in the rat aorta.

Aim 3: Investigate whether the acute presence of tocotrienol rich tocomin can improve endothelium-dependent relaxation of aortae isolated from diabetic and obese rats.

Hypothesis 3: Acute exposure of tocotrienol rich tocomin improves endothelium-dependent relaxation in animal models of type 1 diabetes and obesity in the rat aorta.

Aim 4: Investigate whether the 4 week *in vivo* treatment of diabetic and obese rats with tocotrienol rich tocomin can reduce oxidative stress and improve endothelium-dependent relaxation in the aortae.

Hypothesis 4: *In vivo* treatment of tocotrienol rich tocomin improves endothelium-dependent relaxation in animal models of type 1 diabetes and obesity.

Aim 5: Determine any potential mechanism of action through which tocomin may improve endothelium-dependent relaxation in aortae from diabetic and obese rats.

Hypothesis 5: *In vivo* treatment with tocotrienol rich tocomin improves endothelium-dependent relaxation by increasing the expression of eNOS and decreasing the expression of Nox2 in aortae from diabetic and obese rats.

Chapter 2
General methods

CHAPTER 2: GENERAL METHODS

All procedures involved were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed with the Australian National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes. Numbers of the approvals used throughout the duration of the project were;

1. #0822
2. #1011
3. #1121
4. #1211
5. #1245
6. #1309
7. #1417

Refer to Appendix for Ethics approval.

2.1 Animals

2.1.1 Induction of diabetes and tissue collection.

Male 6-8 week old Wistar rats (240-280g) (Animal Resource Centre, Perth, WA, Australia) were randomly divided into 2 groups: normal and diabetic. The rats were housed in groups of 2-4 under a light/dark cycle (12 h/12 h), in a temperature-controlled room (22°C) at the RMIT Animal Facility with water ad libitum. Type 1 diabetes was induced by a single injection of streptozotocin (STZ, 50 mg/kg) into the tail vein after

an overnight fast. The control groups received an equivalent volume of vehicle (0.1 M citrate buffer, pH 4.5) alone. Induction of diabetes was considered successful when blood glucose levels (BGL) exceeded 15 mM. Bodyweight was measured on a weekly basis to monitor any potential weight loss. BGL was measured weekly using a one-touch glucometer (Roche, Sydney, New South Wales) after obtaining blood from a tail prick. Diabetic rats whose BGL exceeded 15 mM or which had 5% or greater weight loss (compared to initial bodyweight) were administered with long-acting insulin (Protophane; 3-5 units 3 times per week i.p. Novo Nordisk, Australia) to halt any further weight loss and promote health of the rat in the absence of euglycaemia.

The rats were killed 10 weeks post STZ or vehicle injection by asphyxiation by CO_2 inhalation, followed by decapitation and their chests were opened to isolate the thoracic aortae. The rats were fasted overnight prior to the euthanasia. Fasting blood samples were obtained from the carotid arteries following decapitation. Glycated haemoglobin (HbA1c) were measured at the end of the experimental period using the In2it^{TM} A1c system (II) analyser (Bio-Rad, Hercules, CA, USA).

2.1.2 High-fat western diet protocol.

Male Wistar Hooded rats (University of Adelaide, Australia) weighing 180–200 g at the start of feeding period were housed in groups of four under a light/dark cycle (12 h/12 h), in a temperature controlled room (22°C) at the RMIT Animal Facility with water ad libitum. Animals were randomly assigned to either a control diet (SD, Standard AIN93G rodent diet, 7% total fat including 1.05% total saturated fatty acids; Specialty Feeds, Perth, Australia) or high-fat western diet (WD, SF00-219, 21% total fat including 1.80% total saturated fats and 0.15% cholesterol; Specialty Feeds, Perth,

Australia). Animals were allowed adlibitum access to their designated diets for 12 weeks. Food intake and bodyweight were measured weekly. The rats were killed 12 weeks after the feeding period by asphyxiation by CO_2 inhalation, followed by decapitation, and their chests were opened to isolate the thoracic aortae. Blood samples were obtained from the carotid arteries following decapitation. BGL's at the end of the feeding period were measured using a one-touch glucometer (Roche, Sydney, New South Wales). HbA1c was also measured at the end of the feeding period using the In2it^{TM} A1c system (II) analyser (Bio-Rad, Hercules, CA, USA).

2.1.3 Drug administration.

For the study in Chapter 5, six weeks post induction of diabetes or injection of vehicle, tocomin treatment (40 mg/kg/day s.c.) or vehicle (peanut oil) was commenced for a period of 4 weeks until the end of the experimental period. For the western diet rats, 8 weeks into the feeding period tocomin treatment (40 mg/kg/day s.c.) or vehicle (peanut oil) was commenced for a period of 4 weeks until cessation of the study.

2.2 Assessment of vascular function

The thoracic aorta was isolated and immediately placed in ice-cold Krebs bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM D-glucose, and 1.6 mM $CaCl_2$, pH 7.4). The aorta was then cleared of fat and connective tissue and cut into 2-3 mm long segments. The aortic rings were mounted between two stainless steel wires, one of which was linked to an isometric force transducer (model FT03, Grass Medical Instruments, Quincy, MA, USA)

connected to a Powerlab (model 8/30 AD Instrument Co., Sydney, Australia), and the other end anchored to a glass rod submerged in a standard 10 mL organ bath. The organ bath was filled with Krebs-bicarbonate solution. The bath medium was maintained at 37°C, pH 7.4 and continuously aerated with 95% O_2, 5% CO_2. Aortic rings were equilibrated for 45 minutes at a resting tension of 1 g, and then were contracted with an isotonic, high potassium physiological salt solution (KPSS, 122.7 mM KCl, pH 7.4 in which K^+ ions replaced Na^+ ions in the solution) for 20 minutes to achieve maximal contraction. After which KPSS was replaced with krebs solution to re-equilibrate, the rings were sub-maximally contracted with phenylephrine to 40-60 % of KPSS contraction (PE, 0.01–0.3 μM) and endothelial integrity was tested by the addition of a single concentration of acetylcholine (ACh, 10^{-5} M). Where relaxation was greater than 80% of the pre-contraction, the endothelium was considered to be intact and the aortic ring was included in the study. Some additional segments of the thoracic aortae were used to measure superoxide production (Section 2.3).

Cumulative concentration response curves to ACh (0.1 nM–10 μM) and sodium nitroprusside (SNP, 0.1 nM–10 μM) were determined using aortic rings contracted with phenylephrine (10^{-8} to 10^{-7} M) to 40%–60% of maximal contraction. Responses to ACh and SNP were also tested in the presence or absence of inhibitors/antagonists that were added to the baths 20 minutes prior to conducting the response curves. The negative logarithm of the concentration at which 50% relaxation occurred (pEC_{50}) and maximum relaxation (R_{max}) values were calculated from the individual cumulative dose response curves using Graphpad Prism 6.

2.2.1 Basal nitric oxide activity.

Basal NO activity in blood vessels is assessed by measuring basal NO release. Following maximal contraction of the aortae with KPSS, the tissues were washed and relaxed to basal tension and then pre-contracted with PE (10–100 nM) to approximately 20–30% of the maximal KPSS contraction. Under those conditions the further addition of L-NAME (100 μM), a NOS inhibitor, causes further contraction the level of which correlates with the level of the basal release of NO (Chan et al., 2003, Mian and Martin, 1995).

2.3 Chemi-luminescence Assays

Two various chemi-luminessence assays were used to determine superoxide production in vascular tissue and the superoxide scavenging capacity of α-tocopherol, tocomin, and α, γ and δ-tocotrienols in vascular tissue and tissue-free systems.

2.3.1 Lucigenin-enhanced chemi-luminescence tissue-free assay using hypoxanthine/ xanthine oxidase assay.

Superoxide production was measured by lucigenin-enhanced chemi-luminescence using hypoxanthine plus xanthine oxidase as a generator of oxygen radicals. Krebs-HEPES buffer (composition: NaCl 99.90 mM, KCl 4.7 mM, KH_2PO_4 1.0 mM, $MgSO_4.7H_2O$ 1.2 mM, D-glucose 11.0 mM, $NaHCO_3$ 25.0 mM, $CaCl_2 \cdot 2H_2O$ 2.5 mM, Na HEPES 20.0 mM, 300 μL, pH 7.4) containing lucigenin (5mM) and appropriate treatments were placed into a 96-well OptiPlate, followed by the addition of 1unit/mL xanthine oxidase (XO). A background reading was performed after which

hypoxanthine (HX) (10^{-4} M) was added to all wells and superoxide production was measured. Superoxide inhibition was quantified by the superoxide reading subtracted from the background. Results are expressed as a percentage of the counts in the presence of the control.

2.3.2 Lucigenin-enhanced chemi-luminessence superoxide measurement in biological tissue.

Superoxide production in aortic rings was measured using lucigenin-enhanced chemi-luminescence. The principles of this assay are based on methods developed by Guzik and Channon et al. (2005). The methods for this assay are as described by Leo et al. (2011*b*) with the following modification. The thoracic aorta was isolated, cleared of fat and connective tissue, and cut into 2–3 mm long segments which Krebs-HEPES buffer. Aortic ring segments were incubated at 37°C for 45 min in Krebs-HEPES buffer in a Costar cell culture plate in the presence of NADPH (100 mM) as a substrate for NADPH oxidase, DETCA (3 mM), to inactivate endogenous SOD and therefore maximize superoxide detection, either in the absence or presence of a drug (i.e. tocomin, α-tocopherol or α, δ or γ-tocotrienol, etc). In addition superoxide was measured in the presence of diphenylene iodonium (DPI, 5 mM), a flavoprotein inhibitor that inhibits NADPH oxidase, as a positive control for 45 minutes. 300 μL of Krebs-HEPES buffer containing lucigenin (5 mM) and the appropriate treatments were placed into a 96-well Optiplate, and superoxide production was measured. The aortic segments were then transferred to the corresponding wells of the optiplate and the photon emission was recounted. Tissue was dried in a 37°C oven and was weighed. Superoxide production was quantified by normalizing arbitrary units of superoxide production and expressed as a ratio to dry tissue mass (AU/mg dry tissue).

2.3.3 L-012-enhanced chemi-luminessence superoxide measurement in biological tissue.

Superoxide production in aortic rings was also measured using L-012 chemi-luminescence. The principles of this assay are based on methods developed by Miller et al. (2005) with the following modifications. Aortic segments were cleared of fat and connective tissue and cut into 2-3-mm long segments in were incubated at 37°C for 30 min in Krebs-HEPES buffer either alone or in the presence of apocynin, a non specific Nox inhibitor (300 μmol/L) or the general flavoprotein inhibitor diphenylene iodonium (DPI, 5 μM) in a Costar cell culture plate. Krebs-HEPES buffer (300 μL), containing L-012 (100 mM, Wako Pure Chemicals, Osaka, Japan) and the appropriate treatments were in a 96-well Optiplate, which was loaded into the Polarstar Optima plate reader (BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37°C. After background reading was completed, a single aortic segment was added to each well in the optiplate and photon emission was recounted. Superoxide production was quantified by subtracting the final reading from the background reading, superoxide counts were then normalized as arbitrary units of superoxide production and expressed as a ratio to dry tissue mass (AU/mg dry tissue).

2.4 Protein expression

Western blots were performed as described previously by Laemmli (1970) and Woodman and Chan (2004) with the following modifications. For the tocomin diabetic study described in Chapter 5, aortae from three animals from the same treatment group were pooled due to limited availability of tissue, and considered as n = 1. For all others studies in the project, tissue was homogenized individually. The tissues were

homogenized in 200 mL of ice-cold lysis buffer (100 mM NaCl, 10 mM Tris, 2 mM EDTA, 0.5% w/v sodium deoxycholate, 1% v/v Triton X-100, pH 7.4, protease and phosphatase inhibitor cocktails (Roche, Sydney, NSW, Australia). Total protein concentration of the samples was quantified using the Bio-Rad Bradford assay where bovine serum albumin (BSA) was the protein of choice for preparing the standard curve. SDS-PAGE gels prepared where the percentage of acrylamide gels used for western blotting varied according to the molecular mass of the protein of interest (refer to table 2.1). Protein samples were thawed and heated at 95°C for 5 minutes. Equal amounts of protein homogenate were loaded onto the SDS-PAGE gels and ran at 100V till the lowest molecular mass marker was at the bottom of the gel. Proteins in the SDS gel were transferred onto a nitrocellulose membrane (0.45 μm pore size) using a wet transfer at 90V for 90 minutes at 4°C. A successful transfer was confirmed upon Ponceau-S stain. Membranes were blocked in 0.25% BSA in tris-buffered saline with tween 20 (TBST pH 7.4) for 1 hour at room temperature and incubated with anti-mouse/rabbit primary antibodies probing for proteins of interest (all antibody dilutions were 1:1000 in TBST, overnight, 4°C) (refer to Table 2.1). Membranes were washed the following the day (3 x 10 minutes) in TBST followed by incubation in horseradish peroxidase (HRP)-linked mouse/rabbit secondary antibody for 1 hour at room temperature. Membranes were washed and then visualized on the ChemiDoc XRS (Bio-Rad, Sydney, NSW, Australia). All proteins were detected using either enhanced chemi-luminescence (Amersham, GE Healthcare, Sydney, NSW, Australia) or Supersignal West Femto (Thermo Scientific, Rockford, IL, USA). Membranes were then blocked in 0.1% sodium azide (HRP inhibitor) for 1 hour at room temperature and then re-probed with the loading control primary antibody (anti-mouse/rabbit β-actin (1:2000)) and secondary antibody as described above. All protein bands were quantified

by densitometry using ImageLab software (Bio-Rad, Sydney, NSW, Australia) and expressed as a densometric ratio of the primary protein to β-actin.

Table 2.1: Percentage of acrylamide gels used to detect various proteins

Protein	Molecular Mass (k Da)	% Acrylamide gel
Akt	56	10
caveolin-1	22	12
calmodulin	18	12
eNOS	130	7
Nox2	65	10
pAkt (Serine 473)	62	10

2.5 Statistical Analysis and data presentation

All results are expressed as mean±SEM unless stated otherwise, where *n* represents the number of animals per group. Concentration-response curves from the rat isolated aortae were constructed and fitted to a sigmoidal curve using non-linear regression (Graphpad Prism version 6.0, San Diego, CA, USA) to calculate the sensitivity of each agonist (pEC_{50}). Maximum relaxation (R_{max}) to ACh was measured as a percentage of precontraction to phenylephrine. The negative logarithm of the concentration at which 50% relaxation occurred (pEC_{50}) and maximum relaxation (R_{max}) values were calculated from the individual cumulative dose response curves using Graphpad Prism 6. Group pEC_{50} and R_{max} values were compared using a one-way or two-way ANOVA with post-hoc analysis using Sidaks test. $p<0.05$ was considered statistically significant.

Basal NO levels are expressed as the L-NAME contraction as a percentage of the KPSS contraction±SEM. The values were compared using a student *t*-test or a two-way

ANOVA with post-hoc analysis using Sidaks test. $p<0.05$ was considered statistically significant.

Results from superoxide production and antioxidant capacity using hypoxanthine were expressed as a percentage of the counts in the presence of the control±SEM. The level of superoxide inhibition at each concentration was compared to vehicle for each compound using one-way ANOVA with post-hoc multiple comparisons using Dunnet's test. $p<0.05$ was considered statistically significant.

Superoxide levels from rat aortic rings are expressed as arbitrary units (AU) per milligram of dry tissue ±SEM. Results were compared by either a Student's unpaired t-test or one-way ANOVA with a post hoc Dunnett's test as appropriate. $p<0.05$ was considered statistically significant.

All western blotting results were quantified by densitometry using ImageLab software (Bio-Rad, Sydney, NSW, Australia) and expressed as a densometric ratio of the primary protein to β-actin ±SEM. The quantification for the expression of pAkt is expressed as a ratio of pAkt to Akt±SEM.

Chapter 3

The effect of tocotrienols on vascular function in the presence of oxidative stress.

CHAPTER 3: THE EFFECT OF TOCOTRIENOLS ON VASCULAR FUNCTION IN THE PRESENCE OF OXIDATIVE STRESS.

3.1 Introduction

Vitamin E, in addition to the four isoforms of tocopherol, contains four isoforms of tocotrienol. While there has been extensive investigation of the biological activity of the tocopherols there has been much less attention paid to the tocotrienols. There is, however, emerging evidence that the tocotrienols have molecular targets distinct from those of the tocopherols that may result in new therapeutic opportunities (Aggarwal et al., 2010). There are now a number of studies demonstrating cardioprotective actions of tocotrienols. For example, γ-tocotrienol is known to inhibit HMG-CoA reductase and therefore to decrease cholesterol synthesis (Parker et al., 1993). Further, extracts of palm oil, a rich source of tocotrienols, have been demonstrated to activate the NO-cGMP pathway and, as a consequence, to decrease myocardial reperfusion injury (Esterhuyse et al., 2005) perhaps due to scavenging of $ONOO^-$ (Berbee et al., 2011). The antioxidant activity of tocotrienols may also contribute to protective actions in the vasculature (Aggarwal et al., 2010). For example, Newaz and Yousefipour (2003) demonstrated that treatment of spontaneously hypertensive rats with γ-tocotrienol increased NOS activity and lowered arterial pressure, and γ-tocotrienol has also been shown to reduce oxidative stress and inflammation in rats with STZ-induced diabetes (Kuhad and Chopra, 2009). Further Norsidah et al., (2013) reported that a palm oil extract rich in tocotrienols, when orally administered to rats with hyperhomocysteinemia, reduced aortic oxidative stress and increased the plasma level

of NO metabolites. In addition, Muharis et al. (2010) recently demonstrated that a palm oil fraction rich in tocotrienols restored endothelium-dependent relaxation in arteries in rats with STZ-induced type 1 diabetes but it is not clear whether this may have been consequent to a lowering of glucose levels as reported by Budin et al., (2009). There is evidence that the beneficial vascular effects of tocotrienols may extend to man given the report that 2 months treatment with tocotrienols improves pulse wave velocity in healthy males (Rasool et al., 2008).

The mechanism(s) of the beneficial effects of tocotrienols have not been well investigated nor, to the best of our knowledge, has there been any examination of the vascular actions of individual tocotrienol isomers. Therefore, the aims of this study were to compare the antioxidant activity of α-, δ- and γ-tocotrienols with α-tocopherol and tocomin, a palm oil extract rich in tocotrienols (tocotrienol rich fraction: 40%, and palm olein: 38%) but also containing some α-tocopherol (11%). Given the antioxidant activity of these compounds we were further interested to investigate their capacity to protect NO-mediated vascular relaxation as an indication of whether they may be effective in preventing endothelial dysfunction in vascular diseases involving oxidant stress, for example as a result of diabetes (Leo et al., 2011b, Leo et al., 2010b). It has been reported that tocotrienols are incorporated into cellular membranes more rapidly than tocopherol (Saito et al., 2010, Saito et al., 2004, Saito et al., 2003) and that this may contribute to greater antioxidant efficacy. We therefore hypothesized that the tocotrienols would more effectively preserve endothelium-dependent relaxation in the presence of oxidative stress.

3.2 Materials and methods

3.2.1 General protocol for vascular function experiments.

The rats were killed as described in Chapter 2.2. Responses to ACh and SNP were tested in the presence of pyrogallol which is well established to auto-oxidise to generate superoxide and subsequently impair endothelium-dependent relaxation by inactivating NO (Ignarro et al., 1998). Aortae were exposed for 20 minutes to tocotrienol rich tocomin (10^{-6} - 10^{-4} mg/mL), α-tocopherol (10^{-4} - 10^{-2} mg/mL) or tocotrienol isomers (α, δ or γ-tocotrienol (10^{-3} - 10^{-1} mg/mL to determine the effect of varying concentrations of tocomin, α-tocopherol or α, δ or γ-tocotrienol on endothelium-dependent and -independent relaxation in the presence of pyrogallol (30 μM).

Responses to ACh and SNP were also tested in the presence of pyrogallol plus various combinations of α-tocopherol and tocotrienol isomers to replicate tocomin (10% δ-tocotrienol: 20% α-tocotrienol: 50% γ-tocotrienol: 20% α-tocopherol) and other tocotrienol combinations (α+γ)-tocotrienols, and (α+δ+γ)-tocotienols at a concentration of 10^{-4} mg/mL. These experiments were conducted to determine whether an interaction between α-tocopherol and the tocotrienols is necessary to improve endothelium-dependent relaxation in the presence of oxidative stress.

3.2.2 Superoxide generation using hypoxanthine/xanthine oxidase.

The general method for measuring the superoxide scavenging capacity of α-tocopherol, tocomin and tocotrienol isomers is as described in Chapter 2.3.1 with the following modification. Krebs-HEPES buffer (300 μL) containing lucigenin (5 mM) and varying concentrations of α-tocopherol, tocomin and tocotrienol isomers were placed into a 96-

well Optiplate, followed by the addition of 1 unit/mL XO. A background reading was performed after which hypoxanthine (10^{-4} M) was added to all wells and superoxide production was measured. Superoxide inhibition was quantified by the superoxide reading subtracted from the background. Results are expressed as a percentage of the counts in the presence of the control.

3.2.3 Superoxide generation by aorta.

The general method for measuring the superoxide scavenging capacity of α-tocopherol, tocomin and tocotrienol isomers is as described in Chapter 2.3.2 with the following modification. Aortic ring segments were incubated at 37°C for 45 min in Costar cell culture plates in Krebs-HEPES buffer in the presence of NADPH (100 mM), and either alone or in the presence of varying concentrations and combinations of tocomin, α-tocopherol or α, δ or γ-tocotrienol. In addition superoxide was measured in the presence of diphenylene iodonium (DPI, 5 μM), a flavoprotein inhibitor. 300 μL of Krebs-HEPES buffer containing lucigenin (5 mM) and the α-tocopherol, tocomin and tocotrienol isomers were placed into a 96-well Optiplate. A background reading was taken and then aortae segments were transferred to the optiplate in their respective treatment wells and superoxide production was measured and quantified.

3.2.4 Reagents.

All drugs were purchased from Sigma Aldrich except for acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK), tocomin and α, δ and γ-tocotrienols (Carotech, Malaysia). All drugs were dissolved in distilled water, with the exception of tocomin, α-tocopherol and α, δ and γ-tocotrienols that were dissolved in 0.1% DMSO. A mixture

of α-tocopherol and α, δ and γ-tocotrienols which resembles tocomin was prepared (10% δ -tocotrienol: 20% α -tocotrienol: 50% γ -tocotrienol: 20% α –tocopherol), henceforth referred to as the tocomin mixture. Various tocotrienol combinations were also prepared using the following proportions; (α+γ)-tocotrienols (20% α-tocotrienol: 50% + γ-tocotrienol: 30% DMSO) and (α+δ+γ)-tocotienols (10% δ-tocotrienol: 20% + α-tocotrienol: 50% + γ-tocotrienol: 20% DMSO).

3.2.5 Statistical analyses.

Statistical analysis was performed as is described in Chapter 2.5.

3.3 Results

3.3.1 Superoxide scavenging capacity of tocomin, α-tocopherol and α, δ and γ-tocotrienols using hypoxanthine/xanthine oxidase and in rat aorta.

Superoxide production induced by the presence of hypoxanthine/xanthine oxidase is shown in Figures 3.1 and 3.2. α-Tocopherol caused an approximately 50% reduction in superoxide at a concentration of 10^{-2} mg/ml (Figure 3.1A). At the same concentration, all of the tocotrienol isomers caused approximately 80% reductions in superoxide (Figure 3.1C E&G). Tocomin (Figure 3.1I) caused a 50% inhibition of superoxide similar to α-tocopherol but at a concentration 10-100 times lower. The tocomin mixture and various combinations of tocotrienols were also able to scavenge superoxide at similar concentrations to the individual tocotrienol isomers (Figure 3.2A-C). Tocomin was the most effective at scavenging superoxide compared to the tocomin mixture and the various tocotrienol combinations at a concentration 10-100 times lower.

When examined using aorta, all of the compounds of interest were able to significantly reduce superoxide levels but the potency and efficacy was quite different Interestingly, the superoxide scavenging capacity in the aorta was different in comparison to the HX/XO assay. α-Tocopherol and tocomin (Figures 3.2 B&J) produced relatively greater inhibition of aorta-derived superoxide. The tocomin mixture and combinations of tocotrienols were able to quench superoxide but not as effectively as tocomin in the HX/XO assay (Figure 3.2). In contrast, only tocomin and the tocomin mixture were able to scavenge superoxide in the aorta in comparison to the various combinations of tocotrienols (Figure 3.2D). The relative potency between α-tocopherol and tocomin remained the same in this assay with tocomin being equally effective at approximately 100 fold lower concentration.

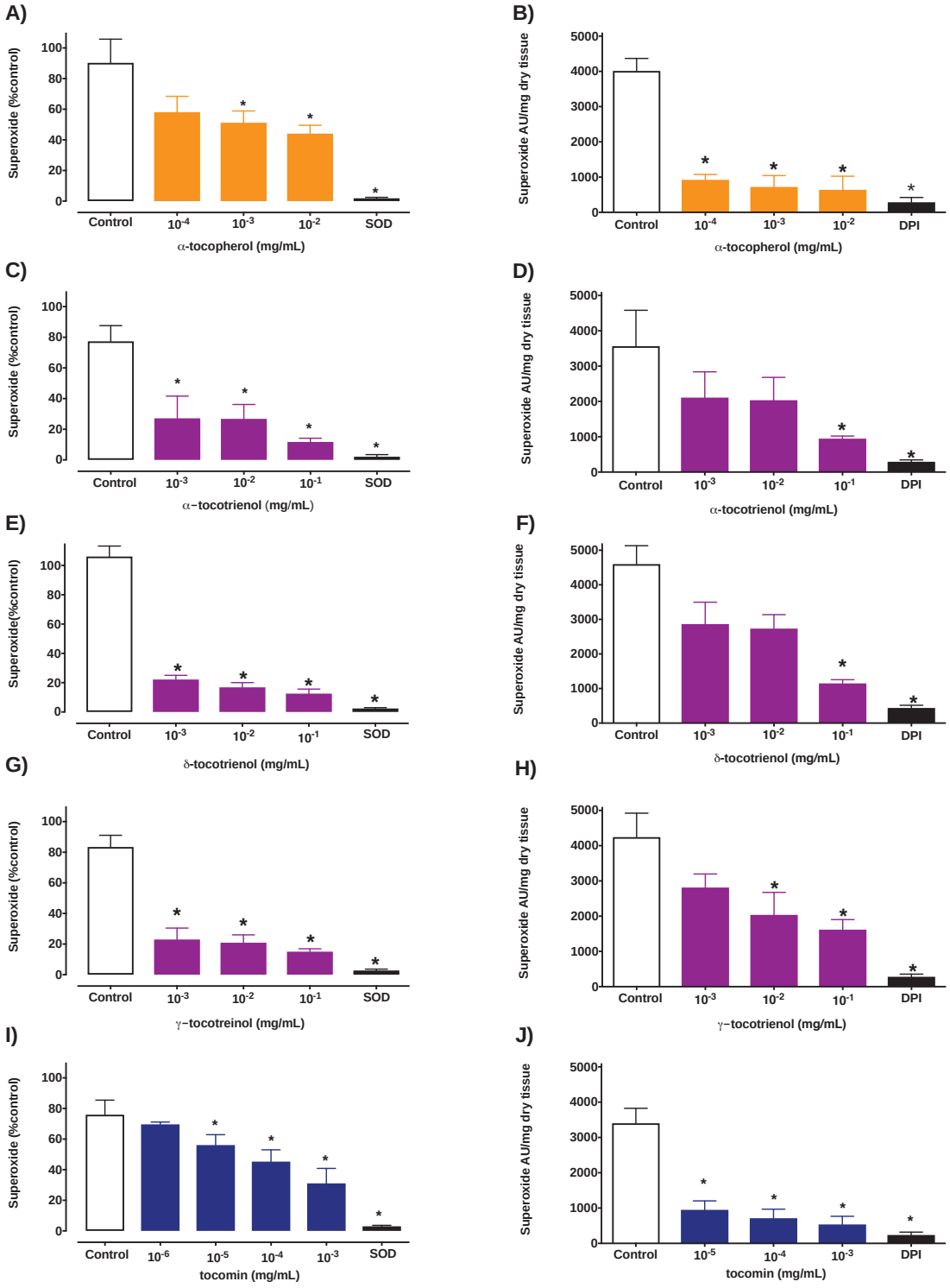


Figure 3.1 Superoxide generated by hypoxanthine (100µM)/xanthine oxidase (0.01U/mL) or rat aorta in the presence of NADPH. *α*-tocopherol (A&B), *α*-tocotrienol (C&D), *δ* -tocotrienol (E&F), *γ* -tocotrienol (G&H), and tocomin (I&J). *Significantly different to control $p<0.05$. Results are shown as mean ± SEM. $p<0.05$. One-way ANOVA. Dunnett's multiple comparisons test. n=3–6 experiments.

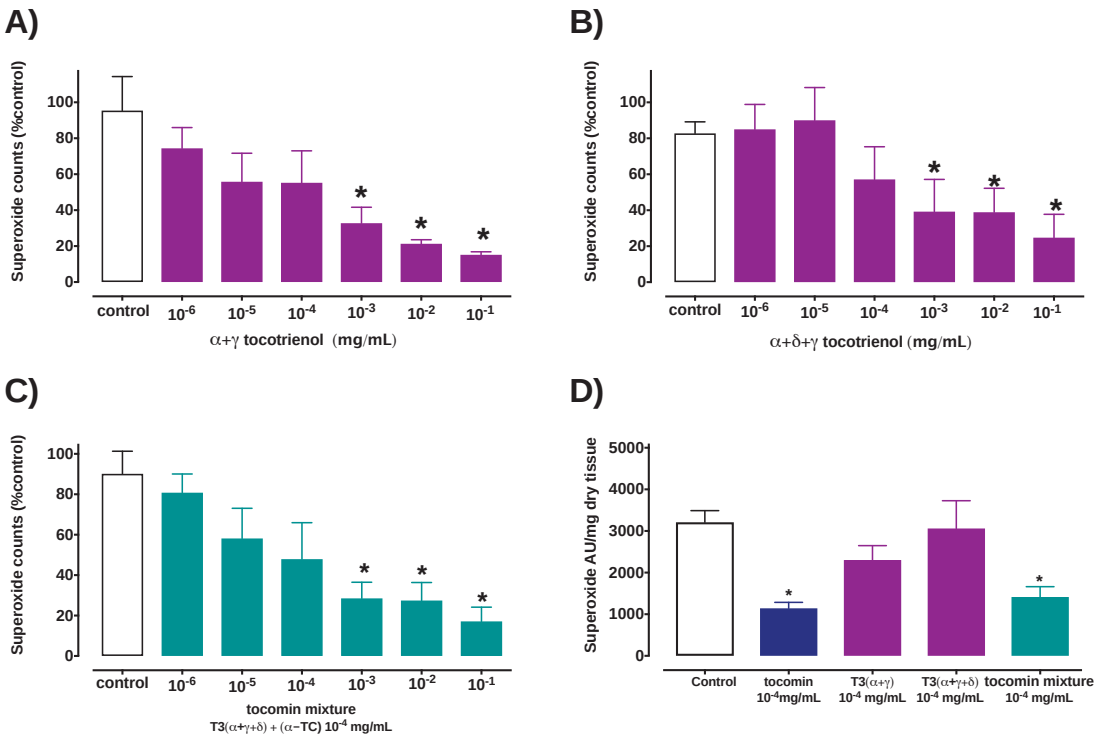


Figure 3.2 Superoxide generated by hypoxanthine (100 μM)/xanthine oxidase (0.01U/mL) (A-C) or in rat aorta in the presence of NADPH (D): α +γ -tocotrienols (A), α+δ+γ -tocotrienols (B), α-tocopherol + (α +γ+δ –tocotrienols) (C), tocomin, tocomin mixture and α +δ +γ – tocotrienols (D). Data is expressed as mean±SEM. *Significantly different to control Results are shown as mean ± SEM. $p<0.05$. One-way ANOVA. Dunnett's multiple comparisons test. n=3–6 experiments.

3.3.2 Vascular function

The effect of pyrogallol-induced oxidative stress and the acute addition of varying concentrations of α-tocopherol, the tocotrienols and tocomin is shown in Figure 3.2. Endothelium-dependent relaxation in response to ACh was significantly inhibited in the presence of pyrogallol-induced oxidative stress with a significant decrease in R_{max} without affecting pEC_{50} (Table 3.1). O_2^- scavenging enzyme SOD, α-tocopherol (Figure 3.3A, 10^{-2} mg/mL) and tocomin (Figure 3.3F, 10^{-4} mg/mL) in the presence of pyrogallol were able to significantly improve endothelium-dependent relaxation however, tocomin improved endothelium-dependent relaxation at a concentration 100 times lower compared to α-tocopherol (Table 3.1). None of the tocotrienol isomers (*α*, *δ* and *γ*-tocotrienols) improved endothelium-dependent relaxation even at concentrations 100 times higher than that of α-tocopherol (Table 3.1). Endothelium-independent relaxation to SNP was not affected by pyrogallol, *α*-tocopherol, tocomin or the tocotrienols (Table 1).

The effect of pyrogallol-induced oxidative stress and the acute addition of varying combinations of α-tocopherol and *α*, δ and *γ*-tocotrienols (10^{-4} mg/mL) is shown in Figure 3.3. Both tocomin and the mixture of (T3(*α*+*δ*+*γ*) + (*α*-TC)) (Figure 3.3E, 10^{-4} mg/mL) significantly improved endothelium-dependent relaxation in the presence of pyrogallol (Table 3.1 Figure 3.3). Other preparations in the absence of *α*-TC, ie. T3(*α*+*γ*) and (*α*+*δ*+*γ*)-tocotienols (10^{-4} mg/mL) did not improve endothelium-dependent relaxation (Figure 3E Table 1). Endothelium independent-relaxation was not affected by the presence of pyrogallol, *α*-tocopherol or *α*+*δ*+*γ*-tocotrienols (Table 1).

Table 3.1: The effect of tocomin, α-tocopherol (TC) and α, δ and γ–tocotrienols (T3) on ACh-induced endothelium-dependent and SNP-induced endothelium-independent relaxation of rat aortae in the presence of pyrogallol-induced oxidative stress.

		ACh		SNP	
	n	pEC_{50} (M)	R_{max} (%)	pEC_{50} (M)	R_{max} (%)
α-tocopherol					
Control	7	7.09±0.18	95±8	8.71±0.10	108±7
Pyrogallol	7	7.13±0.18	47±4$^{\#}$	8.46±0.14	97±5
Pyrogallol + α-TC 10^{-4} mg/mL	5	6.52±0.21	47±5$^{\#}$	8.55±0.12	94±4
Pyrogallol + α-TC 10^{-3} mg/mL	7	6.84±0.12	46±8$^{\#}$	8.35±0.28	96±5
Pyrogallol + α-TC 10^{-2} mg/mL	7	6.97±0.15	70±2*	8.57±0.42	90±5
α-tocotrienol					
Control	5	7.08±0.16	86±10	8.58±0.25	97±3
Pyrogallol	5	6.97±0.15	51±2$^{\#}$	8.67±0.25	96±7
Pyrogallol + α-T3 10^{-3} mg/mL	4	6.25±0.22	47±4$^{\#}$	8.41±0.26	105±4
Pyrogallol + α-T3 10^{-2} mg/mL	5	6.58±0.16	45±5$^{\#}$	8.40±0.09	94±7
Pyrogallol + α-T3 10^{-1} mg/mL	5	6.82±0.23	58±5$^{\#}$	8.12±0.02	105±4
δ-tocotrienol					
Control	5	6.98±0.12	80±3	8.38±0.28	97±4
Pyrogallol	4	6.83±0.45	42±10$^{\#}$	8.49±0.20	99±3
Pyrogallol + δ-T3 10^{-3} mg/mL	4	6.74±0.08	46±9$^{\#}$	8.73±0.07	98±2
Pyrogallol + δ-T3 10^{-2} mg/mL	5	6.75±0.29	43±8$^{\#}$	8.51±0.05	95±8
Pyrogallol + δ-T3 10^{-1} mg/mL	3	6.64±0.30	46±8$^{\#}$	8.60±0.20	99±2
γ-tocotrienol					
Control	6	6.71±0.24	93±4	8.50±0.80	93±4
Pyrogallol	4	6.51±0.15	59±4$^{\#}$	8.45±0.20	98±2
Pyrogallol + γ-T3 10^{-3} mg/mL	4	6.16±0.17	53±4$^{\#}$	8.34±0.12	103±1
Pyrogallol + γ-T3 10^{-2} mg/mL	6	6.71±0.27	55±7$^{\#}$	8.59±0.15	95±4
Pyrogallol + γ-T3 10^{-1} mg/mL	3	6.65±0.22	67±1$^{\#}$	8.68±0.25	97±1
tocomin					
Control	5	7.19±0.08	92±3	8.69±0.18	97±4
Pyrogallol	5	7.06±0.13	58±5$^{\#}$	8.25±0.05	99±2
Pyrogallol + SOD	5	7.10±0.07	88±5*	8.17±0.14	106±4
Pyrogallol + tocomin 10^{-6} mg/mL	4	6.84±0.14	63±6$^{\#}$	8.14±0.24	100±7
Pyrogallol + tocomin 10^{-5} mg/mL	5	6.94±0.13	69±5$^{\#}$	8.23±0.18	103±4
Pyrogallol + tocomin 10^{-4} mg/mL	5	6.70±0.20	81±6*	8.17±0.14	99±2
tocotrienol combinations					
Control	6	7.20±0.15	93±12	8.68±0.15	96±2
Pyrogallol	5	6.77±0.07	50±4$^{\#}$	8.48±0.09	100±2
Pyrogallol + tocomin 10^{-4} mg/mL	5	7.19±0.13	80±3*	8.34±0.16	105±5
P+T3 (α+δ+γ)+ (α-TC) 10^{-4} mg/mL	5	7.02±0.11	73±2*	8.49±0.17	102±2
P + T3 (α+γ) 10^{-4} mg/mL	5	6.86±0.29	65±6$^{\#}$	8.20±0.21	102±1
P + T3 (α+δ+γ) 10^{-4} mg/mL	5	6.57±0.23	61±13$^{\#}$	8.12±0.08	103±2

$^{\#}$Significantly different to control
*Significantly different to pyrogallol
Results are shown as mean±SEM.
One-way ANOVA, $p \leq 0.05$.Sidaks multiple comparison test.

Table illustrates the pEC_{50} and Rmax values for ACh in aortic rings with pyrogallol- induced oxidative stress. The effect of α-tocopherol, tocomin and tocotrienol isomers is shown. n=no. of experiments.

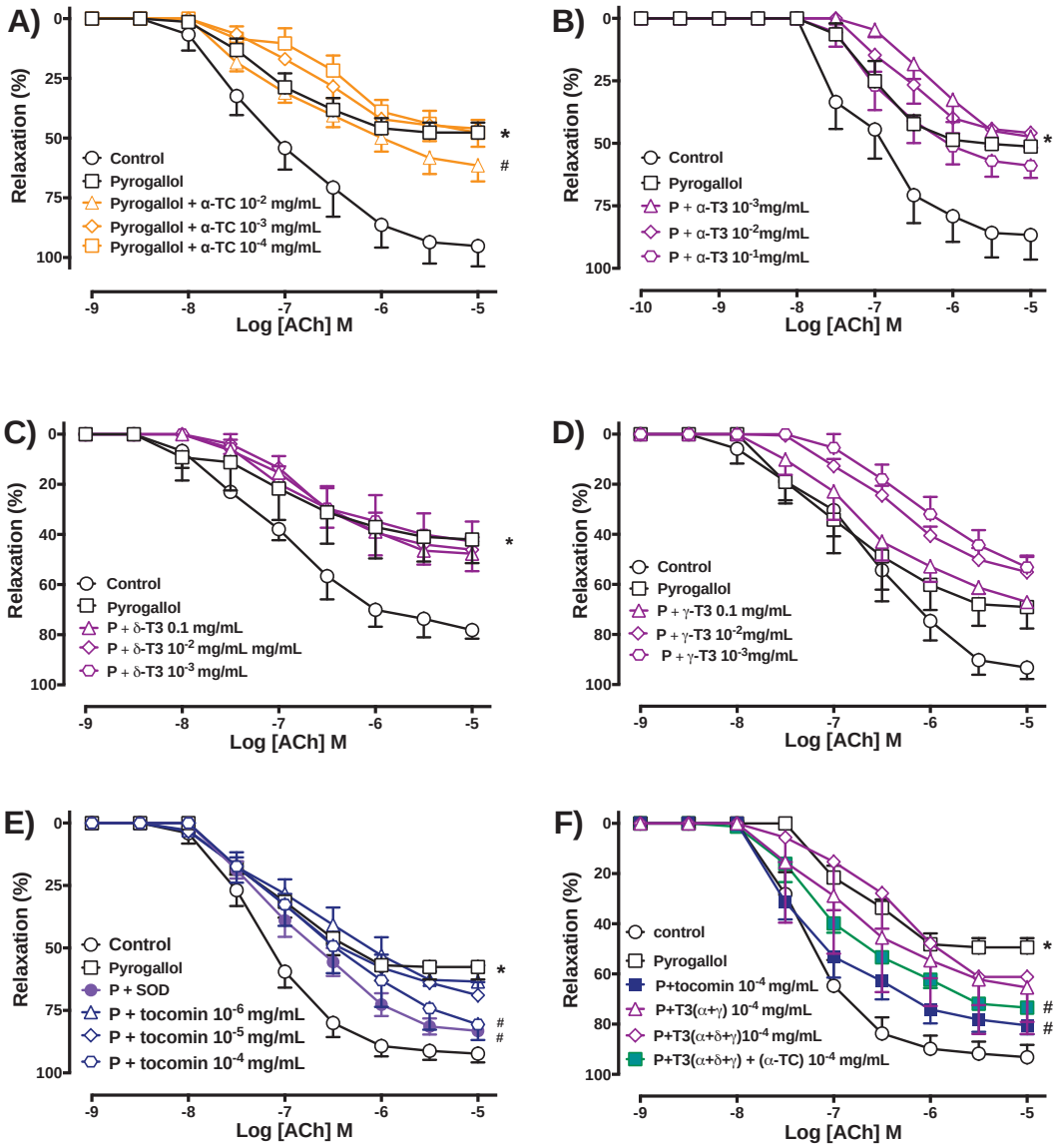


Figure 3.3 Endothelium-dependent and -independent relaxation in rat aortae in the presence of pyrogallol (P): Cumulative concentration-response curves to ACh in the absence (control) or presence of pyrogallol with varying concentrations of *α*-tocopherol (A), *α*-tocotrienol (B), *δ*-tocotrienol (C), *γ*-tocotrienol (D), tocomin (E) and tocotrienol isomers and α-tocopherol as present in the proportions found in tocomin (ie. *α*-T3- 20%, *δ*-T3 10% and *γ*–T3 50% and *α*-TC 20%) (F). *R_{max} Significantly different to control. $^{\#}R_{max}$ Significantly different to pyrogallol. Data is expressed as mean±SEM. $p<0.05$. Sidak's multiple comparison test. *n*=3-7. See Table 3.1 for values and statistical comparison.

3.4 Discussion

This study demonstrated that the tocotrienol isomers were more effective at scavenging superoxide radicals produced by hypoxanthine/xanthine oxidase in comparison to those generated by isolated aortic segments in the presence of NADPH. Tocomin and α-tocopherol restored endothelial function in the presence of oxidative stress but α, δ and γ-tocotrienols were ineffective. α-Tocopherol was less effective than the tocotrienol isomers at similar concentrations when superoxide was generated by hypoxanthine/xanthine oxidase but more effective against superoxide generated by vascular tissue. Tocomin, an extract of palm oil containing predominantly tocotrienols but with some tocopherol, was effective in both assays at 100 fold lower concentrations than α-tocopherol. Consistent with their relatively lower antioxidant activity in isolated vascular tissue, the tocotrienol isomers failed to improve endothelium-dependent relaxation in the presence of oxidant stress. Surprisingly tocomin was the most effective compound at improving endothelium-dependent relaxation and this effect could be replicated by a mixture of α-tocopherol and α, δ and γ-tocotrienols, suggesting that the tocotrienol isomers provide more effective vasoprotection when acting together in combination with α-tocopherol.

In the present study the antioxidant capacity of α-tocopherol, tocomin, α, β, or γ-tocotrienols and various combinations of tocotrienol isomers was examined using hypoxanthine/xanthine oxidase to generate superoxide in a tissue free system or superoxide was produced by NADPH oxidase in segments of rat isolated aorta in the presence of NADPH. These assays have been used previously when testing the antioxidant activity of flavonols as a tool to predict efficacy as vasoprotectants in

vascular disease (Chan et al., 2003). XO is located on blood vessel walls and is an important enzyme that catalyzes the conversion of hypoxanthine to xanthine as a part of purine metabolism producing O_2^- and hydrogen peroxide as a by-product (Cantu-Medellin and Kelley, 2013). XO induced free radical production has been implicated in the pathogenesis of diabetes related vascular complications (Desco et al., 2002). *α*-Tocopherol and *α, δ* and *γ*-tocotrienol were able to scavenge O_2^- at concentrations as low as 10^{-3} mg/mL whereas tocotrienol rich tocomin was able to achieve the same effect at concentrations as low as 10^{-5} mg/mL. Tocopherol and tocotrienols have been demonstrated to exert their antioxidant activity by physically quenching superoxide (Kamal-Eldin and Appelqvist, 1996). Our study that demonstrates that α-tocopherol is 10 times more potent than *α, δ* and *γ*-tocotrienols at scavenging hypoxanthine induced O_2^-. This is surprising given the report by Yoshida et al., (2003) that tocopherol and tocotrienol isomers have a similar antioxidant activity when tested in homogenous solutions. A further surprising observation was that the tocotrienol isomers were less effective at scavenging superoxide derived from the aortic segments as this suggests a limited ability to access the tissue derived reactive oxygen species. This is in contrast to previous observations that tocotrienols are rapidly incorporated into cell membranes which was suggested to be a contributing factor to their antioxidant efficacy (Saito et al., 2003).

The relative antioxidant efficacy of the compounds under examination was different when aortic segments provided the source of superoxide. Tocomin, containing a mixture of tocotrienol isomers and *α*-tocopherol was more effective than the individual isomers and various tocotrienol combinations at reducing oxidative stress whereas in the hypoxanthine/xanthine oxidase assay the opposite situation was observed. The

antioxidant effect of tocomin could be replicated with the tocomin mixture. As noted above this may indicate an increase in activity when the tocotrienol isomers are combined or perhaps there is also an interaction with α-tocopherol.

Our next aim was to investigate whether the compounds could effectively improve endothelium-dependent relaxation impaired by the presence of oxidative stress. Endothelium-derived NO rapidly reacts with O_2^- (rate constant 2×10^{10}M/sec) (Kissner et al., 1997), which reduces its relaxant activity. Superoxide dismutase (SOD) also reacts rapidly with O_2^- (rate constant 1-2 $\times 10^{10}$ M/sec) (Ferrer-Sueta et al., 2002) and in so doing enhances NO bioavailability and may enhance endothelium-dependent relaxation (Jackson et al., 1998). However, antioxidant capacity alone does not guarantee the ability to enhance endothelium-dependent relaxation. For example, the well-known antioxidant ascorbate (vitamin C) does not enhance endothelium-dependent relaxation in arteries when endogenous O_2^- levels are enhanced by inhibiting SOD (Rasool et al., 2008). This is probably due to the relatively slow rate of reaction between ascorbate and O_2^- (2×10^5 M/sec) (Gotoh and Niki, 1992) since exogenous SOD did enhance relaxation. Therefore, one of the aims of this study was to determine whether the tocotrienols scavenged O_2^- rapidly enough to enhance endothelium-dependent relaxation in the presence of basal O_2^- levels and when high concentrations of O_2^- were generated by pyrogallol (Upadhyay et al., 2010). Surprisingly none of the tocotrienols were effective at improving endothelium-dependent relaxation, even at concentrations that decreased detection of superoxide generated by vascular tissue. By contrast, the less effective antioxidant α-tocopherol did improve ACh-induced relaxation. Significantly, tocomin was the compound that most effectively improved endothelium-dependent relaxation in the presence of pyrogallol-induced oxidative

stress. These observations make an interesting comparison to reports that a tocotrienol rich extract was able to acutely improve impaired endothelium-dependent relaxation in aortae removed from spontaneously hypertensive rats or rats with type 1 diabetes caused by STZ Muharis et al., (2010). A third component of tocomin, palm olein consisting mainly of triglycerides, was unlikely to account for the protective actions as it was reported to be without effect in the study by Muharis et al., (2010).

Thus we speculated that the combination of multiple tocotrienol isomers and/or the additional presence of α-tocopherol is necessary to preserve endothelium-dependent relaxation. By testing the preparations with the same proportion of α, δ and γ-tocotrienols and α-tocopherol present in tocomin we determined that only the preparation containing α-tocopherol plus α, δ and γ-tocotrienols preserved endothelial function in the presence of oxidative stress. This data suggests an important interaction between α-tocopherol and tocotrienols to promote protection of vascular function. The mechanism of this positive interaction between α-tocopherol and the tocotrienols is worthy of further investigation.

In conclusion, the capacity of tocomin to preserve endothelium-dependent relaxation has been previously reported with superoxide dismutase preservation of relaxation in the presence of oxidative stress (Ignarro et al., 1998). Whilst α-tocopherol and tocotrienols have been reported to supress signaling processes for example through the inhibition of NF-κB and STAT (Ahsan et al., 2014), the rapid effect seen in this study seems more likely due to antioxidant activity.

3.5 Conclusion

It has been suggested that tocotrienols may have superior antioxidant activity to tocopherols, and we did find that to be true when superoxide is generated by hypoxanthine/xanthine oxidase *in vitro*. By contrast α, δ and γ-tocotrienols and various combinations in the absence of α-tocopherol were largely ineffective in improving NO mediated, endothelium-dependent relaxation in the presence of oxidative stress. However, tocomin, an extract from palm oil rich in tocotrienols and with a minor component of α-tocopherol, was found to be the most effective compound tested. The efficacy of tocomin could be replicated by the presence of α-tocopherol with α, δ and γ-tocotrienols but not by the combined presence of the 3 tocotrienols alone. Thus the combination of tocotrienol isomers and tocopherol may prove to be an effective approach to the preservation of endothelial function where there is disease-induced oxidative stress such as in diabetes and hypertension.

Chapter 4

The effect of acute tocomin on endothelium -dependent relaxation of aortae from diabetic and western diet fed rats.

CHAPTER 4: THE EFFECT OF ACUTE TOCOMIN ON ENDOTHELIUM -DEPENDENT RELAXATION OF AORTAE FROM DIABETIC AND WESTERN DIET FED RATS.

4.1 Introduction

Diabetes is a complex and progressive metabolic disease that is classically diagnosed by hyperglycaemia and disturbances to carbohydrate, protein and fat metabolism due to a marked reduction in the production and efficacy of the hormone insulin (Alberti and Zimmet, 1998). Type 2 diabetes is the most common form of diabetes that accounts for more than 90% of cases worldwide (Chen et al., 2012). The consumption of a high fat western diet has been implicated in the increasing incidence of type 2 diabetes and obesity worldwide (Caballero, 2007).

Both diabetic and obese patients have an increased risk of developing cardiovascular complications (Barton et al., 2012) that is usually preceded by endothelial dysfunction (Pricci et al., 2003). Obesity has become a worldwide pandemic where 39% of adults aged 18 years are obese and in 2013, 42 million children were obese worldwide (WHO, 2015). Obesity is a non-communicable disease that is usually defined as a body mass index (BMI) greater than 30 kg/m^2 (WHO, 2015). It is diagnosed using a combination of measurements including BMI, waist to hip ratio and an accumulation of excess visceral body fat mostly in the abdominal area (Australian Institute Welfare Health, 2015). Obesity can be related to increased dietary energy consumption, alcohol consumption and sedentary lifestyles. Obesity is a classic characteristic of metabolic

syndrome that is defined as a cluster of disorders comprising of hyperglycemia/insulin resistance, obesity and dyslipidemia (Huang, 2009). Metabolic syndrome also increases one's predisposition to diseases such as type 2 diabetes, stroke and CVD (Matsuda and Shimomura, 2013).

Endothelial dysfunction is an established risk factor and predecessor of cardiovascular disorders such as atherosclerosis (Hadi et al., 2005). The vascular endothelium is a thin layer of cells that internally lines all blood vessels and has an important function of regulating vascular tone by producing NO, dihydrogen sulfide (H_2S), prostacyclin and ◌EDH (Figure 1.2). Excessive ROS production can lead to endothelial dysfunction (Bernabé et al., 2013, Vessieres et al., 2013, Leo et al., 2011b). NADPH oxidases (e.g. Nox2) are the major producers of superoxide during diabetes and obesity (De Silva et al., 2011, Marinou et al., 2009). Furthermore, Nox2 driven oxidative stress can cause impairment of endothelium-dependent relaxation through inactivation of NO (Bedard and Krause, 2007).

eNOS is the major producer of NO in the vasculature, whose activity is regulated by several regulatory proteins. Caveolin-1 (cav-1) is an inhibitor of eNOS activity (Li et al., 2014). Phosphorylated Akt (pAkt) is a protein that phosphorylates eNOS, which will subsequently stimulate NO production by phosphorylating eNOS at its Ser1177 site and increase the binding of calmodulin (CaM) to eNOS which is the stimulatory protein for eNOS (Fleming and Busse, 1999).

Due to the increased production of ROS during diabetes and obesity, it can be proposed that antioxidants may serve as a useful therapy to alleviate oxidant stress and hence

reduce cardiovascular complications. Vitamin E is a family of antioxidants, consisting of tocopherols and tocotrienols. The ability of α-tocopherol to improve vascular relaxation in animal models of diabetes has been extensively reported (Guamieri et al., 1996, Keegan et al., 1995). Also, small and large-scale human clinical trials have been performed which have been unable to demonstrate any benefit of vitamin E (α-tocopherol) supplementation in patients with diabetes and cardiovascular disease (Lonn et al., 2005, The Alpha Tocopherol Beta Carotene Cancer Prevention Study Group, 1994). These studies were not performed in diabetic or obese patients exclusively, however they were performed in patients with CVD that have complications of long-term diabetes and obesity. Some suggestions put forward as to why tocopherol supplementation studies have not proven successful in humans include the advanced age of participants, the diabetes and/or vascular disease being too far advanced that treatment with antioxidants such as vitamin E is not beneficial, and the potential for α-tocopherol to become a pro-oxidant at high concentrations (Paravicini and Touyz, 2008, Bowry et al., 1992).

As discussed in the previous chapter amongst the vitamin E family, tocotrienols are a more potent antioxidant than tocopherols" (Serbinova et al., 1991). Also as demonstrated in Chapter 3, tocotrienol rich tocomin is up to 100 times more potent than α-tocopherol in improving vascular relaxation in the presence of pyrogallol-induced oxidative stress. Tocomin is a tocotrienol rich complex whose ability to improve NO mediated endothelium-dependent relaxation or the mechanism through which this may occur in a diabetic or obesity animal model has not been extensively studied. Therefore, based on the findings from Chapter 3 the aim of this study was to investigate whether acute exposure of rat aortae to α-tocopherol and tocomin can improve vascular

relaxation in type 1 diabetic and obesity animal models and whether tocomin is more potent than α-tocopherol in animal models of diabetes and obesity. We also wanted to compare the expression of eNOS, Nox2 and eNOS modulating proteins in the aortae from diabetic and obese rats to compare and contrast the potential mechanisms of endothelial dysfunction in both animal models.

4.2 Materials and methods

4.2.1 Type 1 diabetes.

Male Wistar rats (n=20) were used for this study. The induction of diabetes is as described in Chapter 2.1.1.

4.2.2 Western diet.

Male Wistar-Hooded rats (n=20) were used for this study. The feeding regime and experimental design is as described in Chapter 2.1.2.

4.2.3 Epididymal fat mass.

The epididymal fat of the standard diet (SD) and western diet (WD) rats was collected and weighed at the end of the experimental period. Epididymal fat is composed of white adipose tissue, which is used to store excess body fat, thus making it a good indicator of any changes in the body lipid content (Mathai et al., 2008). The epididymal fat was not measured in the diabetic rats which lost weight in comparison to controls.

4.2.4 General protocol for vascular function experiments.

The rats were killed and the aorta was isolated as described in Chapter 2.2. Responses to ACh and SNP were tested in the presence or absence of the O_2^- scavenging enzyme SOD, α-tocopherol (α-TC, 10^{-2} mg/mL) and tocomin (10^{-4} mg/mL). Responses were also tested in the presence of the eNOS inhibitor L-NNA to investigate the effect of inhibition of eNOS derived NO in the aortae from western diet fed rats. All drugs and inhibitors were added 20 minutes prior to testing vascular function.

4.2.5 Superoxide production in the aorta.

Superoxide production in the thoracic aorta in the presence of tocomin was measured using lucigenin enhanced chemi-luminescence as is described in Chapter 2.3.2.

4.2.6 Basal NO release from aorta.

Basal NO levels that measures KPSS induced contractions was only assessed in the SD and WD rat as is described in Chapter 2.3.1.

4.2.7 Protein Expression

Protein expression (caveolin-1, calmodulin, Nox2, pAkt, Akt and eNOS) was measured using western blotting. The protocols are as described in Chapter 2.4.

4.2.8 Reagents.

All drugs were purchased from Sigma Aldrich except for acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK) and tocomin (Carotech, Malaysia). All drugs

were dissolved in distilled water, with the exception of tocomin and α-tocopherol that were dissolved in DMSO. L-NNA and L-NAME was dissolved in physiological krebs solution. All antibodies were sourced from Genesearch Australia or Merck Millipore (USA).

4.2.9 Statistical Analyses.

Statistical analysis was performed as described in Chapter 2.5.

4.3. Results

4.3.1 Body weights, blood glucose and HbA1c.

The final body weight of the diabetic rats was significantly lower than that of sham rats at the end of the experimental period (Table 4.1). The blood glucose and HbA1c levels of diabetic rats was significantly greater than that of the sham rats (Table 4.1).

The body weight of the western diet fed rats was significantly greater than that of the standard diet fed rats (Table 4.1) at the end of the experimental period. However, the blood glucose and HbA1c levels were not significantly different in the standard diet and western diet fed rats (Table 4.1).

The epididymal fat mass was significantly increased in the WD fed rats compared to the standard diet fed rats as a whole and in proportion to the total bodyweight (Table 4.1).

Table 4.1: Mean body weight, fasting blood glucose, HbA1c levels and epididymal fat mass at the end of the experiment of sham and diabetic and SD and WD rats.

	n	sham	diabetic	n	SD	WD
Final Body Weight (g)	8	415±11	372±17*	8	407±9	464±8^
Blood Glucose (mM)	6	5.6±0.3	27.2±2*	8	5.6±0.3	6.2±0.4
HbA1c (%)	7	5.2±0.1	13±0.3*	7	5.1±0.3	5.1±0.1
Epididymal fat mass (g)	-	*NR*	*NR*	8	8.6±3.9	13.5±0.7^
Epididymal fat (% bodyweight)	-	*NR*	*NR*	8	1.4±0.1	2.4±0.2^

*Significantly different to sham
^Significantly different to SD
NR= not recorded
Results are shown as mean ± SEM
Students unpaired *t* test. p≤0.05.

4.3.2 Superoxide production during diabetes and a high-fat western diet (WD).

Nox-dependant superoxide production was significantly elevated in aortae from both diabetic and western diet fed rats when compared to their respective control. Aortae from diabetic rats that were treated with tocomin (10^{-4} mg/ mL) had significantly reduced Nox-dependant superoxide production. Treatment with the non-selective Nox inhibitor diphenyliodonium (DPI) also attenuated superoxide levels in both groups of rats (Figure 4.1).

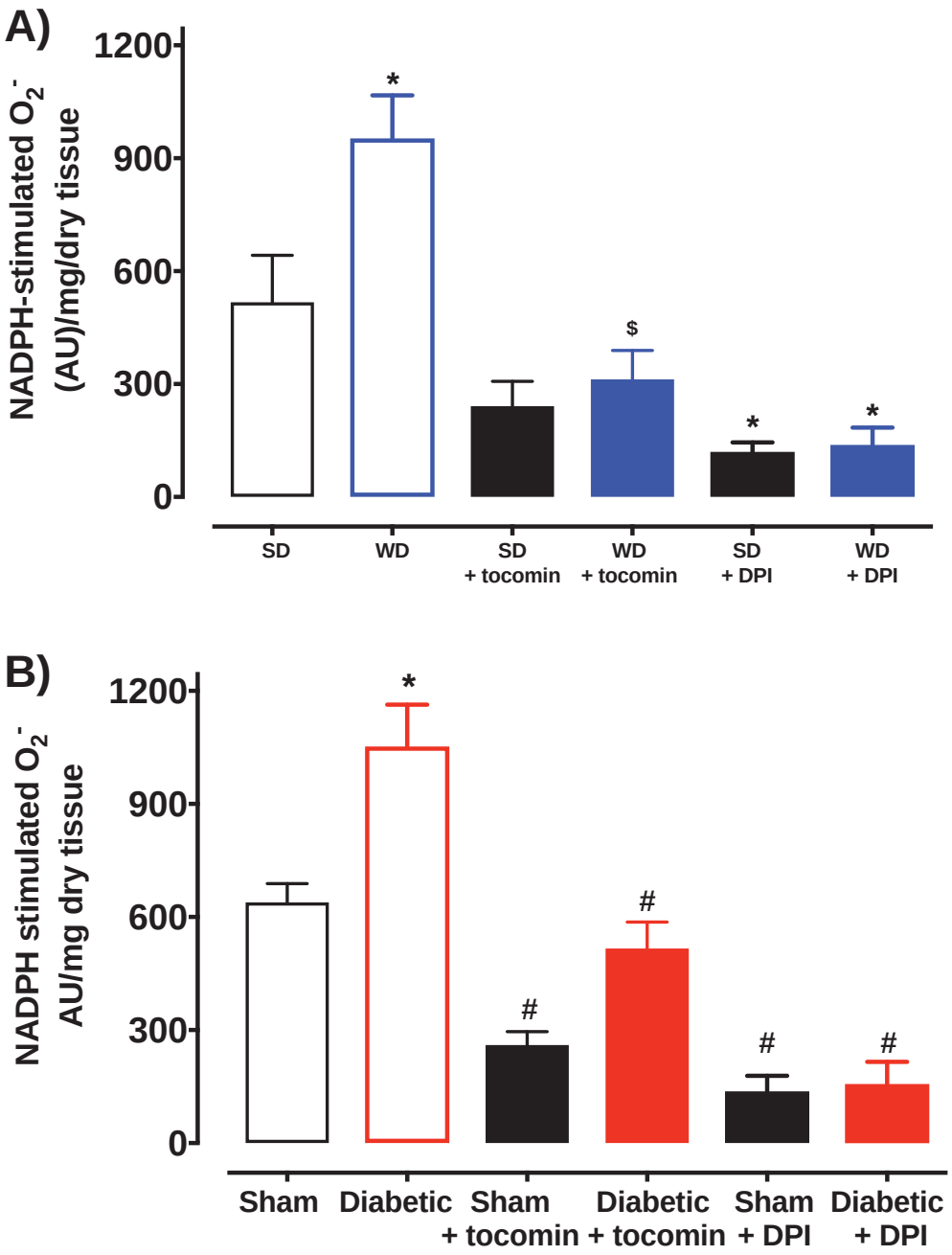


Figure 4.1 Superoxide generated in rat aorta in the presence of NADPH: sham and diabetic rat aortae (A) SD and WD rat aortae (B). $^{\#}$Significantly different to sham. $^{\wedge}$Significantly different to diabetic. *Significantly different to SD. $^{\$}$Significantly different to WD. Results are shown as mean ± SEM. $p<0.05$. One-way ANOVA. Dunnett's multiple comparisons test. n=3–6 experiments.

4.3.3 The effect of a high-fat WD on basal nitric oxide levels.

The contractile response of the aortae to the presence of the eNOS inhibitor L-NAME (100 μM), was significantly decreased in the WD rat aorta in comparison to the SD (Figure 4.2). The contraction in the presence of L-NAME is an indication of basal NO release from the blood vessels. These results indicate that basal NO release from the aortae from the western diet rats is decreased when compared to the aortae from the standard diet fed rats.

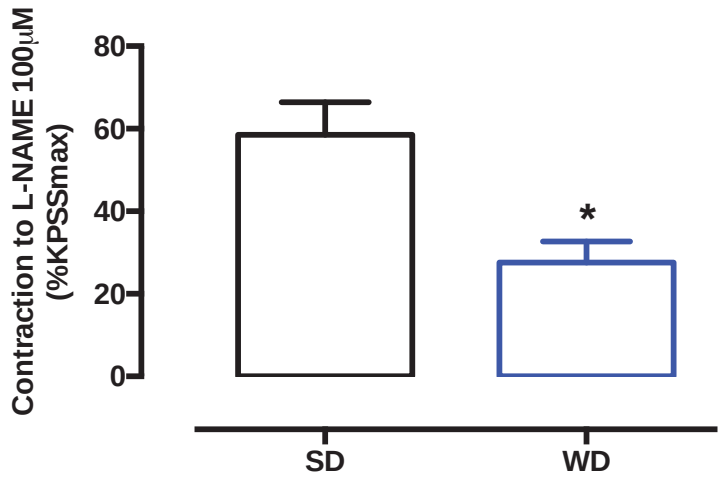


Figure 4.2 Contraction to L-NAME in the SD and WD rat aortae. *Significantly different to SD. Results are shown as mean ± SEM. $p<0.05$. Student's unpaired t-test. $n=10$

4.3.4 The effect of diabetes and a high-fat WD on endothelial function.

Diabetes significantly reduced maximum relaxation (R_{max}) and sensitivity (pEC_{50}) to ACh (Table 4.2; Figure 4.3). Acute exposure to α-TC (10^{-2} mg/ mL) significantly improved maximum relaxation without affecting sensitivity to ACh endothelium-dependent relaxation in diabetic rat aorta (Table 4.2; Figure 4.3). Tocomin (10^{-4} mg/ mL) was also able to significantly improve maximum relaxation (Table 4.2; Figure 4.3)

without affecting sensitivity to ACh (Table 4.2; Figure 4.3) but at a concentration 100 times lower in comparison to α-TC (10^{-2} mg/ mL). Both α-TC (10^{-2} mg/ mL) and tocomin (10^{-4} mg/ mL) were equally effective in improving endothelium-dependent relaxation in the diabetic rat aorta as the major O_2^- scavending enzyme SOD. Endothelium-independent relaxation was not affected by diabetes, α-tocopherol or tocomin (Table 4.2; Figure 4.6A).

Table 4.2: The effect of acute *α*-tocopherol (TC) and tocomin on ACh-induced endothelium-dependent and SNP-induced endothelium-independent relaxation of rat aortae taken from rats with diabetes or a high fat western diet.

	ACh			SNP		
	n	**pEC_{50} (M)**	**R_{max} (%)**	**n**	**pEC_{50} (M)**	**R_{max} (%)**
Sham						
Control	6	7.08±0.08	85±4	7	8.07±0.11	99±3
α-tocopherol 10^{-2} mg/mL	4	7.00±0.08	83±2	6	7.86±0.13	98±2
tocomin 10^{-4} mg/mL	4	7.08±0.11	80±3	6	8.02±0.13	97±2
Diabetic						
Control	6	6.15±0.16*	65±3*	5	7.61±0.26	94±3
SOD	3	7.13±0.40	91±5$^{\$}$	-	*NR*	*NR*
α-tocopherol 10^{-2} mg/mL	6	6.80±0.16	90±2$^{\$}$	4	7.85±0.35	91±6
tocomin 10^{-4} mg/mL	5	6.94±0.21	87±3$^{\$}$	5	7.91±0.37	101±3
Standard Diet (SD)						
Control	7	7.35±0.14	84±4	6	8.48±0.11	94±1
α-tocopherol 10^{-2} mg/mL	3	6.74±0.22	96±6	-	NR	NR
tocomin 10^{-4} mg/mL	5	7.13±0.17	87±4	6	8.31±0.15	95±2
L-NNA	4	6.97±0.26	18±2^	-	*NR*	*NR*
L-NNA + tocomin	4	7.23±0.31	23±3^	-	*NR*	*NR*
Western Diet (WD)						
Control	8	6.80±0.10^	84±4	5	8.41±0.11	96±3
α-tocopherol 10^{-2} mg/mL	4	7.23±0.07$^{\#}$	87±3	-	*NR*	*NR*
tocomin 10^{-4} mg/mL	6	7.27±0.12$^{\#}$	83±5	4	8.22±0.14	97±2
L-NNA	5	6.23±0.27$^{\#}$	12±2$^{\#}$	-	*NR*	*NR*
L-NNA + tocomin	5	6.98±0.10$^{!}$	25±5$^{\#}$	-	*NR*	*NR*

NR= not recorded
*Significantly different to control (sham)
$Significantly different to control (diabetic)
^ Significantly different to control (SD)
#Significantly different to control (WD)
! Significantly different to L-NNA (WD)
Results are shown as mean±SEM.
One-way ANOVA, $p<0.05$. Sidaks multiple comparison test.

Table illustrates the pEC_{50} and Rmax values for ACh in aortic rings from standard diet (SD), western diet (WD), sham and diabetic rats. The effect of α-tocopherol, tocomin and L-NNA is shown. n=no. of experiments.

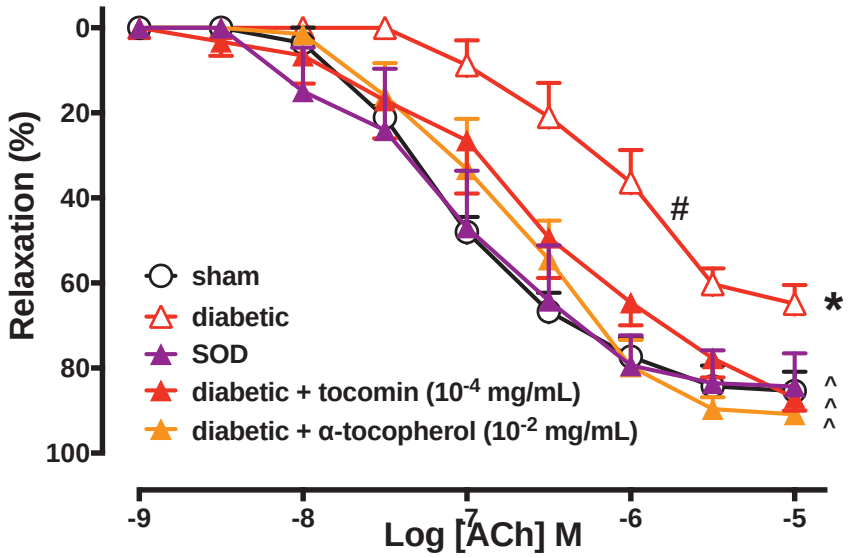


Figure 4.3 Cumulative concentration–response curves to ACh in the absence or presence of α-tocopherol, tocomin or SOD (1000U) in endothelium-intact aortae isolated from sham and diabetic rats. $^{\#}pEC_{50}$ Significantly different to sham. $*R_{max}$ Significantly different to sham. $\hat{}R_{max}$ Significantly different to diabetic. Data is expressed as mean±SEM. $p<0.05$. Sidak's multiple comparison test. *n*=3-7. See Table 4.2 for values and statistical comparison.

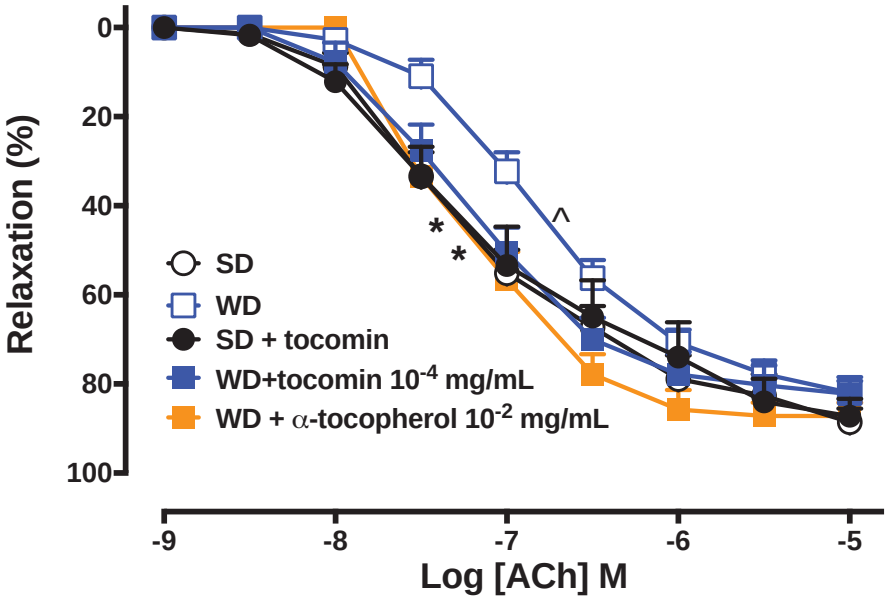


Figure 4.4 Cumulative concentration–response curves to ACh in the absence or presence of α-tocopherol (10^{-2} mg/ mL) and tocomin (10^{-4} mg/ mL) in endothelium-intact aortae isolated from SD or WD rat aortae. $\hat{}pEC_{50}$ Significantly different SD. $*pEC_{50}$ Significantly different WD. Data is expressed as mean±SEM. $p<0.05$. Sidak's multiple comparison test. *n*=4-8. See Table 4.2 for values and statistical comparison.

WD significantly reduced sensitivity to ACh (pEC_{50}) without affecting maximum relaxation in the aortae (Table 4.2; Figure 4.4). α-TC (10^{-2} mg/ mL) significantly improved sensitivity to ACh but did not affect maximum relaxation in WD rat aorta (Table 4.2; Figure 4.4). Acute exposure to tocomin (10^{-4} mg/ mL) also significantly improved ACh sensitivity without affecting maximum relaxation (Table 4.2; Figure 4.4). In the presence of the eNOS inhibitor L-NNA, maximum relaxation and sensitivity to ACh was significantly decreased in both SD and WD rat aortae (Table 4.2; Figure 4.5B). Acute treatment of the WD aortae with tocomin (10^{-4} mg/ mL) in the presence of L-NNA significantly increased sensitivity to ACh in comparison to the SD without improving maximum relaxation (Table 4.2; Figure 4.5B).

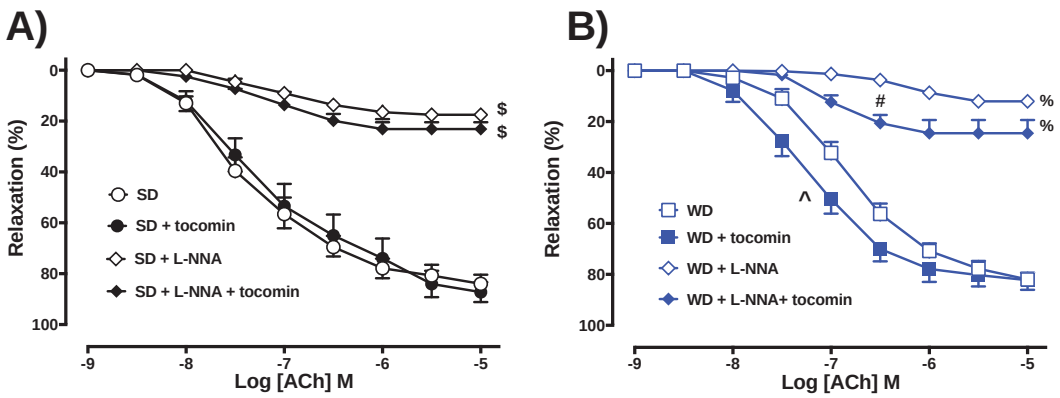


Figure 4.5 Cumulative concentration–response curves to ACh in the absence or presence of tocomin (10^{-4} mg/mL) and L-NNA in endothelium-intact aortae isolated from SD and (A) WD (B) rats. $^{\$}R_{max}$ Significantly different to SD. $^{\%}R_{max}$ Significantly different to WD. $^{\wedge}pEC_{50}$ Significantly different to WD. $^{\#}pEC_{50}$ Significantly different to WD + L-NNA. Data is expressed as mean±SEM. $p<0.05$. Sidak's multiple comparison test. n=3-8. See Table 4.2 for values and statistical comparison.

The response to SNP was not affected by diabetes, a western diet, α-tocopherol or tocomin (Figure 4.6) indicating that smooth muscle function was not affected by diabetes, a western diet, α-tocopherol and tocomin.

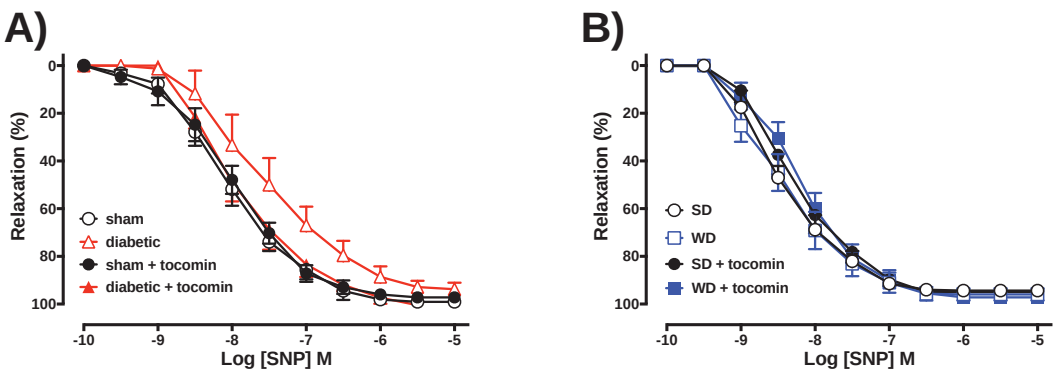


Figure 4.6 Cumulative concentration–response curves to SNP in the absence or presence of α-tocopherol (10^{-2} mg/mL) or tocomin (10^{-4} mg/mL) in endothelium-intact aortae isolated from sham and diabetic and (A), SD and WD (B) rats. Data is expressed as mean±SEM. Sidak's multiple comparison test. *n*=3-8. See Table 4.2 for values and statistical comparison.

4.3.5 The effect of diabetes and a western diet on Nox2, eNOS, and modulatory proteins.

The expression of the superoxide-producing enzyme Nox2 was significantly increased in aortae from both diabetic and WD rats in comparison to the sham and SD rats respectively (Figures 4.7 A&B). The total expression of the NO producing enzyme eNOS was also significantly lower in aortae from diabetic and WD rats in comparison to the sham and SD rats (Figures 4.7 C&D). In addition, in WD rat aortae the expression of calmodulin (CaM) (Figures 4.8D) and ratio of phosphorylated Akt (pAkt) to Akt at the Ser473 site was decreased in comparison to the SD rat aortae (Figures 4.8B). The expression of CaM and pAkt/Akt was not affected by diabetes (Figures 4.8 A&C). Caveolin-1 expression was increased in the WD rat aorta in comparison to SD rats (Figure 4.8F). The expression of cav-1 was not affected by diabetes (Figure 4.8E).

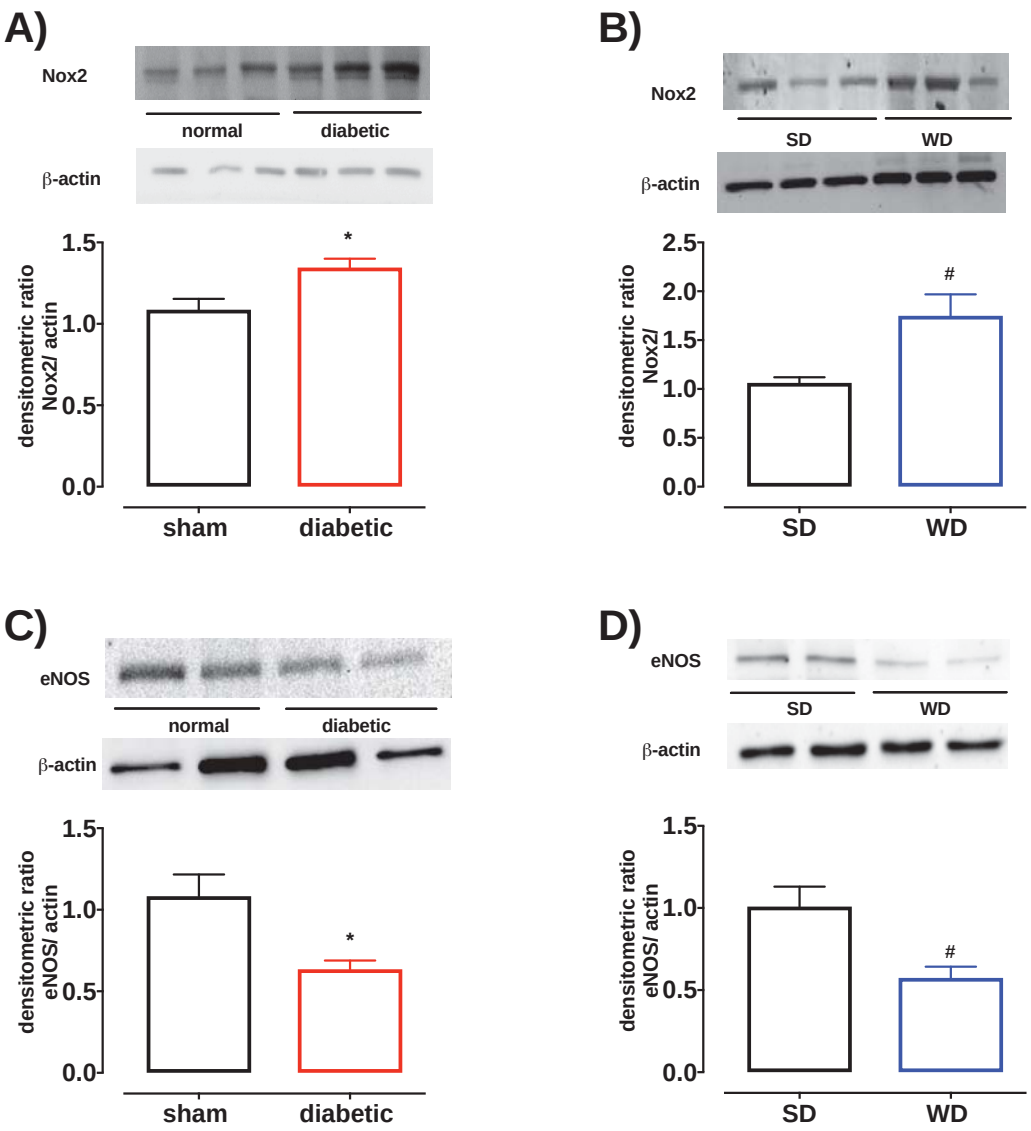


Figure 4.7 Protein expression of NADPH oxidase (Nox2; A&B) total endothelial NOS (eNOS; C&D) from sham /diabetic and SD/WD rat aortae. Representative blots are shown for each corresponding graph. *Significantly different to sham. #Significantly different to SD. Results are shown as mean ± SEM, $p<0.05$. Student's unpaired t-test; $n = 3–5$ experiments.

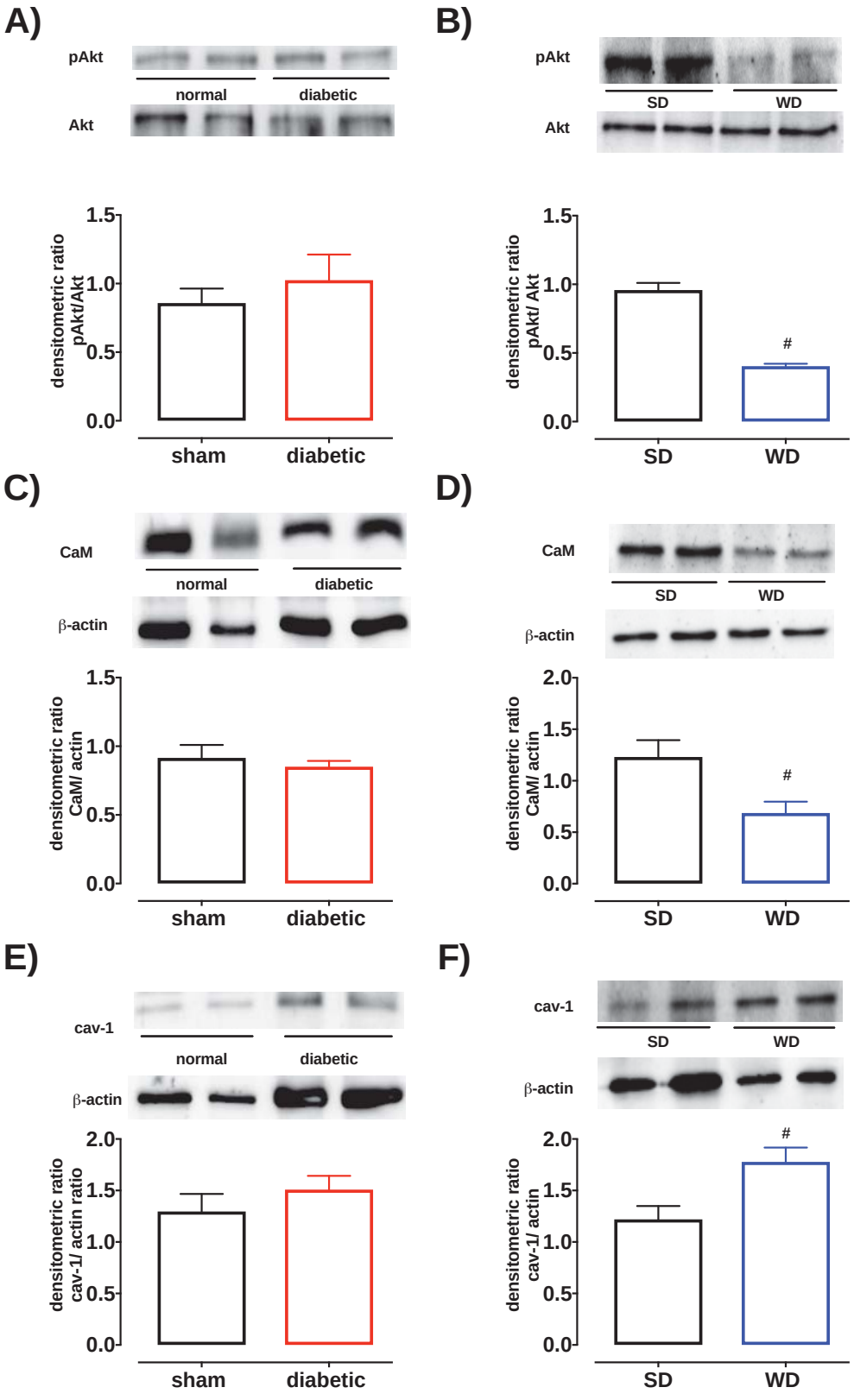


Figure 4.8 Protein expression of pAkt/Akt (A&B) calmodulin-1 (C&D) and caveolin-1 (E&F). Representative blots are shown for each corresponding graph. Results are shown as means ±SEM; *Significantly different to sham. #Significantly different to SD. Results are shown as mean ± SEM, $p<0.05$. Student's unpaired t-test; $n = 3–5$ experiments.

4.4 Discussion

There is substantial evidence in the literature that diabetes and a high-fat western diet causes oxidative stress and contributes to the impairment of endothelium-dependent relaxation in the vasculature. This study examined the effect of acutely exposing aortae from type 1 diabetic and high-fat western diet fed rats to α-tocopherol and tocotrienol rich tocomin and its effect on oxidative stress and vascular function.

4.4.1 The effect of diabetes and a high-fat WD on oxidative stress and endothelium-dependent relaxation.

When comparing the metabolic factors of the 2 animal models diabetes caused a significant increase in BGL and HbA1c in comparison to the sham rats. The high-fat WD did not cause any increase in blood glucose levels or HbA1c at the end of the study. The type 1 diabetic rats lost weight in comparison to the sham rats whereas the WD group had a significantly higher body weight and epididymal fat mass proportionate to bodyweight at the end of the feeding period (Table 4.1). This is similar to the observation previously made when using the same diet (Kosari et al., 2012). Epididymal fat mass provides an established indication of obesity (Soret et al., 1974). Both diabetes and a WD caused a significant increase in the O_2^- generated by the aortae (Figure 4.1 A&B) and this correlated with an increase in the expression of the NADPH oxidase subunit Nox2 (Figure 4.7 A&B). Nox2 is the major O_2^- producing enzyme in the vasculature (Paravicini and Touyz, 2008) and the association between increased oxidative stress and increased Nox2 expression in the vasculature during diabetes and a high fat diet and CVD has been previously reported (Wang et al., 2016, Joshi and

Woodman, 2012, Malakul et al., 2008). Acute treatment of the aortae with tocotrienol rich tocomin was able to significantly reduce the O_2^- in the diabetic and WD aortae (Figure 4.1). This demonstrates that tocomin is an effective antioxidant. The ability of tocotrienols to reduce oxidative stress induced protein damage has been previously demonstrated by Adachi and Ishii (2000). These results also correlate with the observations in Chapter 3 where acute tocomin treatment of rat aortae subjected to oxidative stress was able to reduce O_2^- production. The WD-induced oxidative stress was accompanied by a decreased contractile response to the eNOS inhibitor L-NAME and impaired endothelium dependent relaxation to ACh (Figures 4.2 & 4.5). Although basal NO levels from the diabetic aortae would have been useful to measure in this study they were not measured due to a lack of aortic tissue however, it has been previously demonstrated that under these conditions NO bioavailability is significantly reduced (Leo et al., 2012).

4.4.2 The effect of diabetes and a high-fat WD on endothelial function.

At the end of the experimental period the diabetic rat aorta had endothelial dysfunction that was seen as impaired responses to ACh in comparison to the sham rats (Table 4.2; Figure 4.3) without any changes to SNP. In contrast the diabetic rat aortae, the WD rat aorta also demonstrated endothelial dysfunction in comparison to the SD rat aorta however the WD aortae only had reduced sensitivity to ACh and no impairment of maximum relaxation (Table 4.2; Figure 4.4). The observations from this study are interesting because they are indicative that a WD causes selective impairment of endothelial function in the rat aortae as has been previously reported (Jenkins et al.,

2016, Wang et al., 2016). Both diabetic and WD rat aortae also had a decreased eNOS expression (Figure 4.7 C&D). This observation is interesting as it indicates that despite decreased expression of major NO producing enzyme eNOS and increased Nox2 expression in both diabetic and WD rat aortae, the manifestation of endothelial dysfunction is different during diabetes and obesity. A possible explanation for the varying mechanisms in both the diabetic and WD rat aortae will be discussed later.

4.4.3 The effect of acute α-tocopherol and tocomin exposure on endothelial function during diabetes and a high-fat WD.

The acute treatment of the aortae from diabetic and the WD fed rats (Figure 4.4) rat aortae with α-tocopherol (10^{-2} mg/mL) and tocotrienol rich tocomin (10^{-4} mg/mL) significantly improved endothelium-dependent relaxation but tocomin was more effective at a concentration 100 times lower than α-tocopherol. The effectiveness of tocotrienols as antioxidants in comparison α-tocopherol has not been extensively studied. For example, Serbinova et al., (1991) demonstrated in rat microsomal membranes with NADPH-induced lipid peroxidation, α-tocotrienol to be 40-60 times more potent as an antioxidant in comparison to α-tocopherol. They also demonstrated α-tocotrienol to have higher physiological activity than α-tocopherol under conditions of oxidative stress due to its high recycling efficiency in the presence of ascorbic acid (vitamin C).

In the presence of L-NNA maximum relaxation and sensitivity to ACh was significantly reduced (Table 4.2; Figure 4.5) in both the SD and WD aortae. This demonstrates that eNOS makes a major contribution to ACh-induced relaxation in this blood vessel. With the addition of tocomin in the presence of L-NNA there was a significant increase in

sensitivity to ACh in the WD aortae (Figure 4.5B). This suggests that acutely tocomin may increase NO bioavailability through a greater contribution of a non-eNOS derived source of NO or that tocomin may stimulate a greater contribution of EDH-type relaxation. The contribution of an EDHF to enhance endothelium-dependent relaxation has been demonstrated by Ellis et al., (2008) where a high fat diet did not cause endothelial dysfunction in LDLR-/- rats. This was attributed to an increase contribution of EDH-type relaxation.

Another interesting observation in this study was that in the absence of diabetes in a high-fat western diet and in the presence of endothelial dysfunction the aorta could still maintain maximum relaxation. In contrast, in the diabetic aorta there is decreased sensitivity and maximum relaxation to ACh (Figures 4.3 & 4.4). This could indicate that in the presence of oxidative stress and in the absence of elevated blood sugar level e.g. obesity there may be a greater contribution of a compensatory mechanism eg: prostacylins that may be playing a greater role to compensate any further loss of any vascular function. However, this would require further investigation to elucidate any potential compensatory mechanism of endothelium-dependent relaxation in the obese rat aorta. Endothelium-independent relaxation was not affected by diabetes, a western diet, α-tocopherol or tocomin. (Table 4.2; Figure 4.6).

4.4.4 The effect of diabetes and a high-fat WD on eNOS modulatory proteins.

When comparing the expression of eNOS and its modulatory proteins in the diabetic and obese rat aorta we may be able to elucidate the potential mechanisms of endothelial dysfunction.

pAkt stimulates eNOS by phosphorylating it at the Ser1177/1179 site in response to various stimuli such as shear stress (Zhao et al., 2015, Dimmeler et al., 1999, Fleming and Busse, 1999). The ratio of pAkt to Akt indicates the level of Akt-induced eNOS activation (Dimmeler et al., 1999). Our study demonstrated that diabetes did not affect pAkt/Akt expression however total eNOS expression was still decreased (Figures 4.7 & 4.8). Therefore, these results suggest that a decrease in Akt-induced eNOS activation is not the cause for endothelial dysfunction in the aorta during diabetes rather there is another mechanism that is causing endothelial dysfunction and a decrease in eNOS expression in the diabetic rat i.e. increased O_2^- production.

During states of increased oxidative stress such as diabetes there can be up-regulation of other physiological pathways e.g. the polyol pathway (Giacco and Brownlee, 2010). These pathways can lead to the depletion of vital cofactors that are essential for NO production such as NADPH and BH_4 (Chen et al., 2008). This can cause in eNOS to uncouple into a monomeric state (Satoh et al., 2005). In this case O_2 can become an electron acceptor rather than L-arginine therefore making eNOS behave in a similar manner to Nox2 further exaggerating O_2^- production (Triggle and Ding, 2010). Hence, it can be hypothesized that the decreased eNOS expression seen in this study in the diabetic rat aortae is due to the uncoupling of eNOS that has resulted from an increase in oxidative stress. This may lead to subsequent CaM-induced eNOS activation, however this would require further investigation.

In contrast to the diabetic rat aorta in the WD aortae a decrease in pAkt/ Akt expression was accompanied with a decrease in total eNOS expression. These results indicate that in the presence of oxidative stress there is a decrease in eNOS expression and Akt-

induced eNOS activation in the WD rat aortae however a greater contribution of an EDH allows maximum relaxation to be maintained in the presence of a decrease in the sensitivity to ACh.

CaM and cav-1 expression was also investigated in the study. CaM and cav-1 are regulatory proteins of eNOS where CaM is stimulatory to eNOS that binds to eNOS in response to increase Ca^{2+} concentrations and cav-1 is inhibitory that binds to eNOS in response low increase Ca^{2+} concentrations (Zhao et al., 2015, Fleming and Busse, 1999). CaM and cav-1 expression was not altered in the diabetic rat aorta in comparison to the sham aorta (Figure 4.8C&E). Unaltered CaM and cav-1 expression I diabetic aorta are not consistent with similar studies. A study that was conducted by Joshi and Woodman (2012) demonstrated that following 6-weeks of type 1 diabetes in the presence of increased oxidative stress, endothelium-dependent relaxation was preserved in the rat aorta. This was due to an increased expression of eNOS and CaM expression that increased NO bioavailability therefore preserving vascular function. However, in this study following 10-weeks of diabetes, in the rat aortae there was absolute endothelial dysfunction with no effect on the expression of eNOS regulatory proteins cav-1, CaM, pAkt and AKt. Therefore this study suggests that in advanced diabetes endothelial dysfunction is potentially due to decreased NO bioavailability that is a result of increased oxidative stress and increased Nox2 expression and any possible compensatory mechanisms e.g. EDHF is not sufficient to preserve endothelial function.

In contrast to the diabetic aortae CaM expression was decreased and cav-1 expression was increased in the WD rat aortae in comparison to the SD (Figure 4.8D&F). As mentioned previously CaM and cav-1 are complimentary to each other's function i.e.

cav-1 is inhibitory to eNOS and CaM is stimulatory to eNOS. These results suggest that CaM and cav-1 play a greater role in endothelial dysfunction in the obese rat aorta in comparison to the diabetic rat aorta. pAkt promotes eNOS activity by the phosphorylation of Ser1177/Ser1179 and Thr495, among other key sites (Siragusa and Fleming, 2016, Wang et al., 2016) thus promoting the binding of CaM to eNOS. This study suggests that the decrease in CaM expression in the WD aorta observed in this study could potentially due be to decreased Akt-eNOS phosphorylation, however eNOS phosphorylation was not investigated in this study. Also, the decrease in eNOS expression could be due to an increase in oxidative stress coupled with an increase in Nox2 expression.

Increased cav-1 expression has been associated with obesity and CVD where caveolins especially cav-1 has been associated with regulating lipid levels and free cholesterol transport (Martin and Parton, 2005). A study conducted by Cohen et al., (2004) demonstrated that increased cav-1 expression is associated with increased lipid accumulation in mice. Another studied conducted by Feng et al., (2003) demonstrated increased cholesterol loading in the ER of foam cells was associated with increased cav-1 expression. Accumulation of cholesterol in foam cells form fatty streaks in the vasculature that is a hallmark characteristic of atherosclerosis (Martin and Parton, 2005). Cav-1 expression in the adipocytes of the WD rats was not measured in this study however this study demonstrates that increased cav-1 expression is associated with obesity that was seen as in increase in epididymal fat mass and contributes to endothelial dysfunction in the aorta of a high-fat fed rats and increases oxidative stress in the aorta of the WD rat aortae.

4.5. Conclusion

In conclusion, this study demonstrated that both diabetes and a high-fat western diet cause endothelial dysfunction in the diabetic and WD aortae in comparison to the sham and SD aortae respectively. Diabetes causes reduced sensitivity to ACh and reduced maximum relaxation in the rat aortae in comparison, a WD reduced sensitivity to ACh but was able to achieve maximum relaxation. Endothelial dysfunction can be attenuated acutely in the diabetic and WD rat aorta by the addition of α-tocopherol (10^{-2} mg/mL) and tocomin (10^{-4}mg/mL), hence making tocomin up to 100 times more potent than α-tocopherol. These results are consistent with our findings in Chapter 3 where acute exposure to tocomin and α-tocopherol was able to improve vascular function during pyrogallol-induced oxidative stress. We have also demonstrated that there is a decrease in NO bioavailability in WD rat aortae in comparison to SD aortae that correlates with endothelial dysfunction in the WD rat aorta.

Similarly O_2^- production was increased in both the diabetic and WD rat aortae that was associated with increased Nox2 expression. The acute treatment of the aortae with tocomin was able to attenuate O_2^- production in both diabetic and WD rat aortae. Endothelium-dependent relaxation was almost completely abolished in the presence of the eNOS inhibitor L-NAME indicating that endothelium-dependent relaxation in the WD aortae is almost entirely mediated through eNOS derived NO. Sensitivity to ACh in the presence of L-NAME can be improved with the acute presence of tocomin without improving maximum relaxation suggesting the possible mechanism through which acute exposure to tocomin improved endothelium-dependent relaxation in the

WD aortae is through a non-eNOS source of NO or a greater contribution of EDHF-type relaxation.

This study also demonstrates the mechanisms of endothelial dysfunction in the diabetic and WD rat aortae in comparison to the sham and SD aortae respectively. Increased O_2^- production in the diabetic and WD aortae can be attributed to increased Nox2 expression. Both diabetic and WD aortae also had decreased eNOS expression. However during diabetes the expression of pAkt/ Akt is not affected, neither is the expression of CaM and cav-1 suggesting that in advanced diabetes (10-weeks) pAkt/Akt, cav-1 or CaM do not provide any compensatory mechanism to improve endothelium-dependent relaxation. Also due to the increased oxidative stress resulting from increased Nox2 expression and the up-regulation of other O_2^- producing pathways eg; the polyol pathway and mitochondrial activity, may potentially be causing the uncoupling of eNOS resulting in a decreased NOS expression hence further exacerbating oxidative stress and endothelial dysfunction. In contrast in the WD aorta in the presence of oxidative stress, decreased eNOS functionality was associated with decreased Akt activity as demonstrated by a decreased pAkt/Akt ratio and also a decrease in CaM expression. This study has also demonstrated that increased cav-1 expression in the WD rat aortae contributes to endothelial dysfunction during obesity.

4.6 Acknowledgements

We would like to thank Dr Joanne Hart for providing the experimental work and data for Figure 4.2.

Chapter 5

The effect of 4-week tocomin treatment on endothelium-dependent relaxation in aortae from diabetic and western diet fed rats.

CHAPTER 5: THE EFFECT OF 4-WEEK TOCOMIN TREATMENT ON ENDOTHELIUM-DEPENDENT RELAXATION IN AORTAE FROM DIABETIC AND WESTERN DIET FED RATS.

5.1 Introduction

The increasing incidence of diabetes and obesity in western societies is clearly correlated with the incidence of CVD (Grundy, 2004). There is growing evidence that a contributing mechanism to the development of diabetes and obesity-induced cardiovascular disease is an associated increase in oxidative stress (Furukawa et al., 2004). A well-characterized trigger for the initiation and progression of vascular disease is an impairment of endothelial function. Feeding rats and mice a "western" diet (WD), that is, a diet with an elevated level of total and saturated fats, leads to impairment of endothelium-dependent relaxation (Jenkins et al., 2016, Wang et al., 2016, Roberts et al., 2005). The impairment of endothelium-dependent relaxation is strongly linked to diabetes- and obesity -induced increases in oxidative stress due to increased synthesis of superoxide anions in the vasculature.

There has been considerable interest in the potential use of vitamin E to reduce cardiovascular disease but largely with disappointing outcomes from clinical trials (Mathur et al., 2015, Schmidt et al., 2015). Most clinical trials have focused specifically on the actions of α-tocopherol, the vitamin E form found most abundantly in mammalian tissues (Rigotti, 2007), but vitamin E has three additional isoforms of tocopherol (β, γ, δ) and four tocotrienols (α, β, γ, δ). There is growing evidence that

tocotrienols may exert biological activities similar to, but also distinct from, the tocopherols (Peh et al., 2016, Aggarwal et al., 2010).

When investigating the capacity of tocotrienols to preserve endothelial function in the presence of oxidative stress in vitro we found that when present as individual isomers α-, δ-, and γ-tocotrienol failed to demonstrate any beneficial effect (Chapter 2). Importantly, this lack of preservation of endothelium- dependent relaxation was despite the demonstration of effective antioxidant activity in a separate assay. This contrasted with the ability of tocomin, a palm oil extract containing a high concentration of tocotrienols and a lesser component of tocopherol, to significantly improve endothelium-dependent relaxation in the presence of oxidative stress. Crucially, the protective actions of tocomin could be replicated by a mixture of tocotrienols with, but not without, tocopherol. Thus, we came to the conclusion that a mixture of tocotrienols and tocopherol may provide more effective protection of endothelial function against oxidative stress than any of the individual components in isolation. We also have demonstrated that acute tocomin exposure in diabetic and high fat fed WD rat aortae can improve vascular function (Chapter 4). Therefore the aim of the present study was to investigate whether the acute protective actions of tocomin can be replicated when subcutaneously administered in vivo to rats where oxidative stress is induced by diabetes and the consumption of a high-fat WD that causes endothelial dysfunction.

5.2 Materials and methods

5.2.1 Animals.

All procedures were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed to the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes.

5.2.2 Type 1 diabetes.

Male Wistar rats were used for this study. The induction of diabetes and experimental design is as described in Chapter 2.1.1.

5.2.3 Western diet.

Male Wistar-Hooded rats were used for this study. The feeding regime and experimental design is as described in Chapter 2.1.2.

5.2.4 Epididymal fat mass.

The epididymal fat of the standard diet (SD) and western diet (WD) rats was collected and weighed at the end of the experimental period. Epididymal fat is composed of white adipose tissue, which is used to store excess body fat, thus making it a good indicator of any changes in the body lipid content. The epididymal fat was not measured in the diabetic rats because the rat weight was not significant and this information would be of no scientific benefit.

5.2.5 Drug administration.

Six weeks post induction of diabetes or injection of vehicle, tocomin treatment (40 mg/kg/day s.c.) or vehicle (peanut oil) was commenced for a period of 4 weeks until the end of the experimental period. For the Western Diet rats, 8 weeks into the feeding period tocomin treatment (40 mg/kg/day s.c.) or vehicle (peanut oil) was commenced for a period of 4 weeks until cessation of the study. The dosage of tocomin was established following an extensive literature review. There are previous studies that used tocotrienol rich fractions with dosage ranging from 5 g/kg/day- 200mg/kg/day (Bayorh et al., 2005, Budin et al., 2009). There was also a study that demonstrated γ-tocotrienol to enhance NO activity in blood vessels from spontaneously hypertensive rats with doses ranging (15-150 mg/kg/day) (Newaz and Yousefipour, 2003). Therefore, because tocomin has a 40% tocotrienol content, the aim of our dose of choice was to have a total tocotrienol content that is lower than 15 mg/kg/day to determine whether a tocotrienol rich fraction can achieve a similar effect.

5.2.6 General protocol for vascular function experiments.

Cumulative concentration–response curves to ACh and SNP were determined using aortic rings contracted with PE to 40–60% of maximal contraction. Responses to ACh and SNP were also tested in the presence or absence of a small calcium activated potassium-channel (SK_{Ca}) inhibitor apamin (1 μM), intermediate calcium activated potassium-channel (IK_{Ca}) channel inhibitor TRAM-34 (1 μM), nitric oxide synthase (NOS) inhibitor, N-nitro-l-arginine methyl ester (L-NAME, 100 μM), and soluble guanylate cyclase (sGC) inhibitor, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM) to investigate the role of NO and endothelium-dependent

hyperpolarization (EDH) through the opening of potassium channels in the relaxant responses. All treatments were added to the baths 20 min prior to conducting the concentration–response curves. The negative logarithm of the concentration at which 50% relaxation occurred (pEC_{50}), and maximum relaxation (*R*max) values were calculated from the individual cumulative concentration response curves using Graphpad Prism 6.

5.2.7 Superoxide production in aorta.

Superoxide production in the sham and diabetic thoracic aorta was measured using lucigenin-enhanced chemi-luminescence as is described in Chapter 2.3.2. Superoxide production in the SD and WD thoracic aorta was measured using L-012-enhanced chemi-luminescence as is described in Chapter 2.3.3.

5.2.8 Protein expression.

Protein expression was performed using western blotting. The protocol is as described in Chapter 2.4.

5.2.9 Reagents.

All drugs were purchased from Sigma Aldrich except for acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK) and tocomin (Carotech, Malaysia). All drugs were dissolved in distilled water, with the exception of tocomin that were dissolved in peanut oil and L-NNA/ L-NAME was dissolved in physiological krebs solution. All antibodies were sourced from Genesearch Australia or Merck Millipore (USA).

5.2.10 Statistical analyses

Statistical analysis was performed as is described in Chapter 2.5.

5.3 Results

5.3.1 The effect of diabetes and tocomin treatment on body weights, blood glucose and HbA1c.

The body weight of sham rats was significantly greater than that of diabetic rats (Table 5.1) at the end of the experimental period. The BGL and HbA1c levels of diabetic rats was significantly greater than that of the sham rats (Table 5.1). 4-week treatment had no effect on bodyweight, BGL or HbA1c levels (Table 5.1)

5.3.2 The effect of diabetes and tocomin treatment on superoxide production in the rat aorta.

Nox2 superoxide production was significantly elevated in diabetic rat aortae in comparison to the sham rat aorta. 4-week treatment of the WD rats with tocomin (40 mg/kg/day) was able to significantly attenuate superoxide production in the tocomin treated diabetic rat aortae. Treatment with DPI, a non-selective inhibitor of NADPH oxidase also decreased superoxide levels in the aorta from standard diet and western diet fed rats (Figure 5.1).

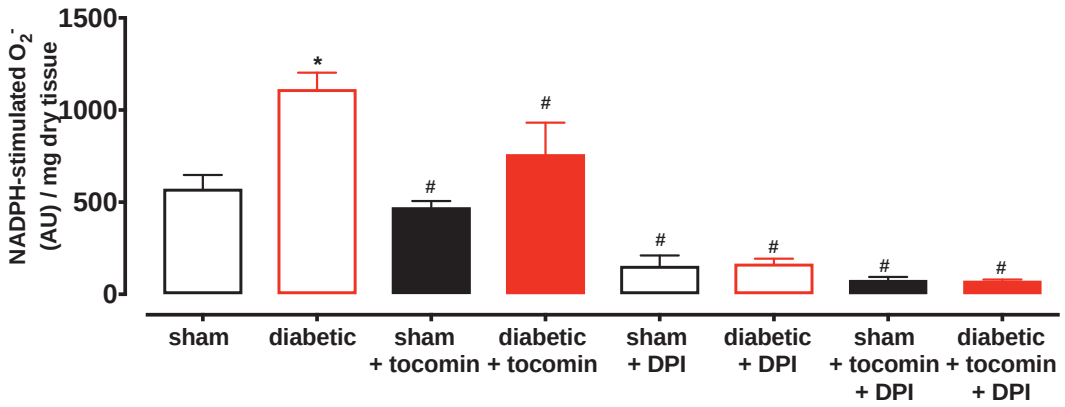


Figure 5.1 Superoxide generated in rat aorta in the presence of NADPH from sham, diabetic, tocomin treated (sham+tocomin/diabetic+tocomin) and in the presence of DPI. Data is expressed as mean±SEM. *Significantly different to sham. #Significantly different to diabetic. Results are shown as mean ± SEM. $p<0.05$. Two-way ANOVA, Dunnett's multiple comparisons test. n=3–6 experiments.

Table 5.1: Mean body weight, fasting blood glucose and HbA1c levels at the end of the experimental period for sham, diabetic and tocomin treated (sham + tocomin/diabetic + tocomin) rats.

	n	sham	n	diabetic	n	sham + tocomin	n	sham + tocomin
Final Body Weight (g)	10	522 ± 17	7	381 ± 22*	7	552 ± 17	8	374 ± 30*
Blood Glucose (mM)	9	6.8 ± 0.3	7	32.2 ± 1.2*	8	7.4 ± 0.7	7	29.3 ± 3.1*
HbA1c (%)	9	5.7 ± 0.2	7	13.3 ± 0.3*	7	5.5 ± 0.2	7	11.8 ± 1.3*

*Significantly different to sham
Results are shown as mean±SEM.
Two-way ANOVA, $p<0.05$.

5.3.3 Effect of diabetes and tocomin treatment on endothelial function.

The maximum response (R_{max}), but not the sensitivity (pEC50), to the endothelium-dependent dilator ACh was significantly reduced in the aortae from diabetic compared to sham rats (Table 5.2; Figure 5.2A). Responses to SNP were not affected by diabetes (Table 5.2; Figure 5.2B). The 4-week treatment of the diabetic rats with tocomin (40 mg/kg/day sc) significantly improved the maximum response to ACh in the aorta from diabetic, but not sham, rats. Tocomin treatment did not affect the endothelium-independent relaxation to SNP in any group.

In the presence of inhibitors of SK_{Ca} (apamin) and IK_{Ca} (TRAM- 34), ACh-induced relaxation is mediated by NO. The maximum response to ACh was significantly decreased in the diabetic rat aorta compared to the sham aorta (Table 5.2; Figure 5.2C). Following 4-week treatment with tocomin, there was no change in ACh-sensitivity in aortae from the sham rats, but the maximum response to ACh was significantly increased in the diabetic rat aorta by the tocomin treatment. Tocomin treatment had no effect on the endothelium-independent relaxant responses to SNP in any group (Table 5.2; Figure 5.2D). This suggests that tocomin treatment improves endothelial release of NO from the diabetic rat aorta rather than influencing sensitivity to either endogenous or exogenous NO.

In the presence of the eNOS inhibitor L-NNA alone, the maximum relaxation to ACh was significantly decreased in comparison to the sham in the absence of L-NNA however in the presence of L-NNA maximum relaxation was significantly deceased in the sham rat aortae in comparison to the diabetic aortae (Table 5.2; Figure 5.2E). These

results indicate the predominant contribution of NO to endothelium-dependent relaxation in this large artery and the minor contribution of a non-NO mediated mechanism eg: prostacyclin or EDHF. In the presence of the sGC inhibitor ODQ alone, ACh-induced relaxation was not different in the aortae from diabetic rats compared to sham rats (Table 5.4; Figure 5.3A), and tocomin did not affect responses in either groups. These results suggest that in both the shamand diabetic rat aortae eNOS derived NO is the main cause of endothelium-dependent relaxation and 4-week treatment of WD rats with tocomin does not improve maximum relaxation through an eNOS/sGC-independent mechanism. The presence of TRAM-34 and apamin, in addition to L-NNA and ODQ, did not further attenuate responses to ACh in any group (Table 5.4; Figure 5.3C) indicating an absence of contribution of calcium-activated potassium (K_{Ca}) channels to endothelium- dependent relaxation in this large artery.

L-NNA did not significantly affect responses to SNP (Figure 5.2F). However, ODQ did significantly reduce the sensitivity and maximum response to SNP in aortae from both sham and diabetic rats (Table 5.2; Figure 5.3D). Tocomin did not affect responses to SNP under any of the tested conditions.

Table 5.2: The effect of 4-week tocomin treatment on ACh-induced endothelium-dependent and SNP-induced endothelium-independent relaxation of rat aortae during diabetes.

		ACh		SNP	
	n	**pEC_{50} (M)**	**R_{max} (%)**	**pEC_{50} (M)**	**R_{max} (%)**
Sham (vehicle)					
control	7	6.95±0.23	93±4	8.42±0.15	97±3
tram + apamin	5	7.27±0.21	91±5	8.38±0.40	97±1
L-NNA	3	6.51±0.26*	16±3*	8.21±0.27	92±5
ODQ	8	6.51±0.26*	8±2*	6.00±0.30	28±8
L-NNA + ODQ + tram + apamin	7	*ND*	0*	5.88±0.36	58±6
Diabetic (vehicle)					
control	7	6.75±0.13	76±4$	8.54±0.17	93±3
tram + apamin	5	6.67±0.06	72±4$	8.80±0.29	100±2
L-NNA	3	6.73±0.21	28±4*	8.71±0.16	103±2
ODQ	5	6.73±0.29	10±4*	6.58±0.24	48±5
L-NNA + ODQ + tram + apamin	4	*ND*	0*	6.96±0.28	34±8
Sham + tocomin (40 mg/kg/day)					
control	7	6.93±0.09	83±4	8.36±0.25	96±2
tram + apamin	3	6.79±0.10	82±5	8.10±0.20	100±2
L-NNA	6	6.88±0.13	23±8*	8.50±0.26	96±5
ODQ	7	6.90±0.06	11±7*	5.95±0.20	48±8
L-NNA + ODQ + tram + apamin	6	*ND*	0*	5.81±0.20	33±9
Diabetic + tocomin (40mg/kg/day)					
control	7	7.38±0.16	89±3#	8.54±0.14	101±4
tram + apamin	5	7.1±0.10	80±5	8.56±0.33	94±3
L-NNA	6	6.68±0.16	38±5*	8.47±0.23	100±3
ODQ	7	6.80±0.21	16±7*	6.26-±0.48	48±8
L-NNA + ODQ + tram + apamin	5	*ND*	3± 3*	5.83±0.28	43±8

ND = not determined
*Significantly different compared to control in the same treatment group
$ Significantly different compared to the same treatment in the sham group
# Significantly different to same treatment in the diabetic group
Results are shown as mean±SEM.
Two-way ANOVA, $p<0.05$. Sidaks multiple comparison test.

Table illustrates the pEC_{50} and Rmax values for ACh in aortic rings from sham, diabetic and tocomin treated (sham+tocomin/diabetic+tocomin) rats. The effect of various chemical inhibitors is shown. n=no. of experiments.

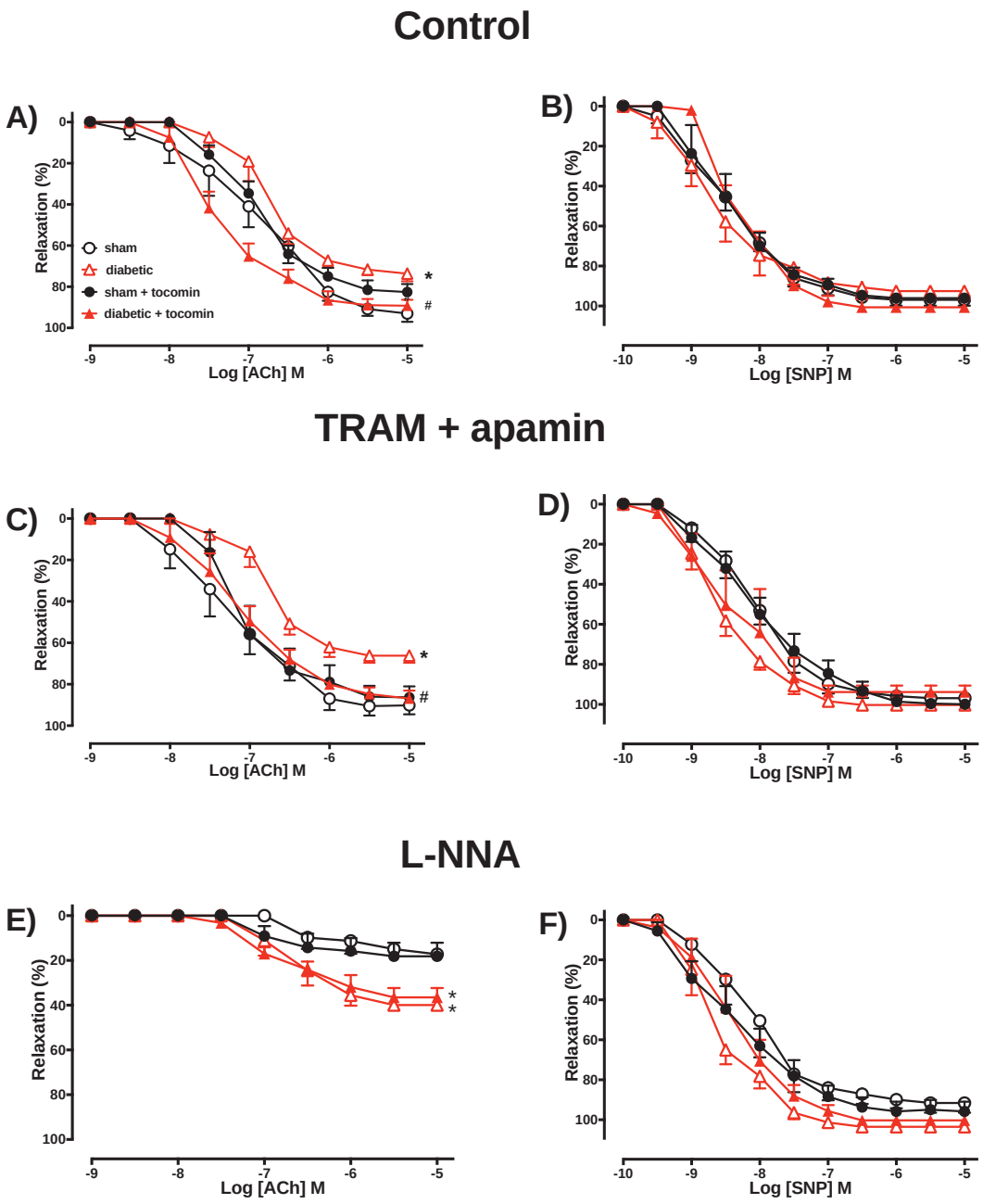


Figure 5.2 Cumulative concentration–response curves to ACh and SNP in endothelium-intact aortae isolated from sham diabetic, and tocomin treated (sham + tocomin/diabetic + tocomin) rats in control (A&B) or the presence of Tram + apamin (C&D), and L-NNA (E&F). $^{*}R_{max}$ Significantly different to sham. $^{\#}R_{max}$ Significantly different to diabetic. $p<0.05$. Data is expressed as mean±SEM. Sidak's multiple comparison test. *n*=3-7. See Table 5.2 for values and statistical comparison.

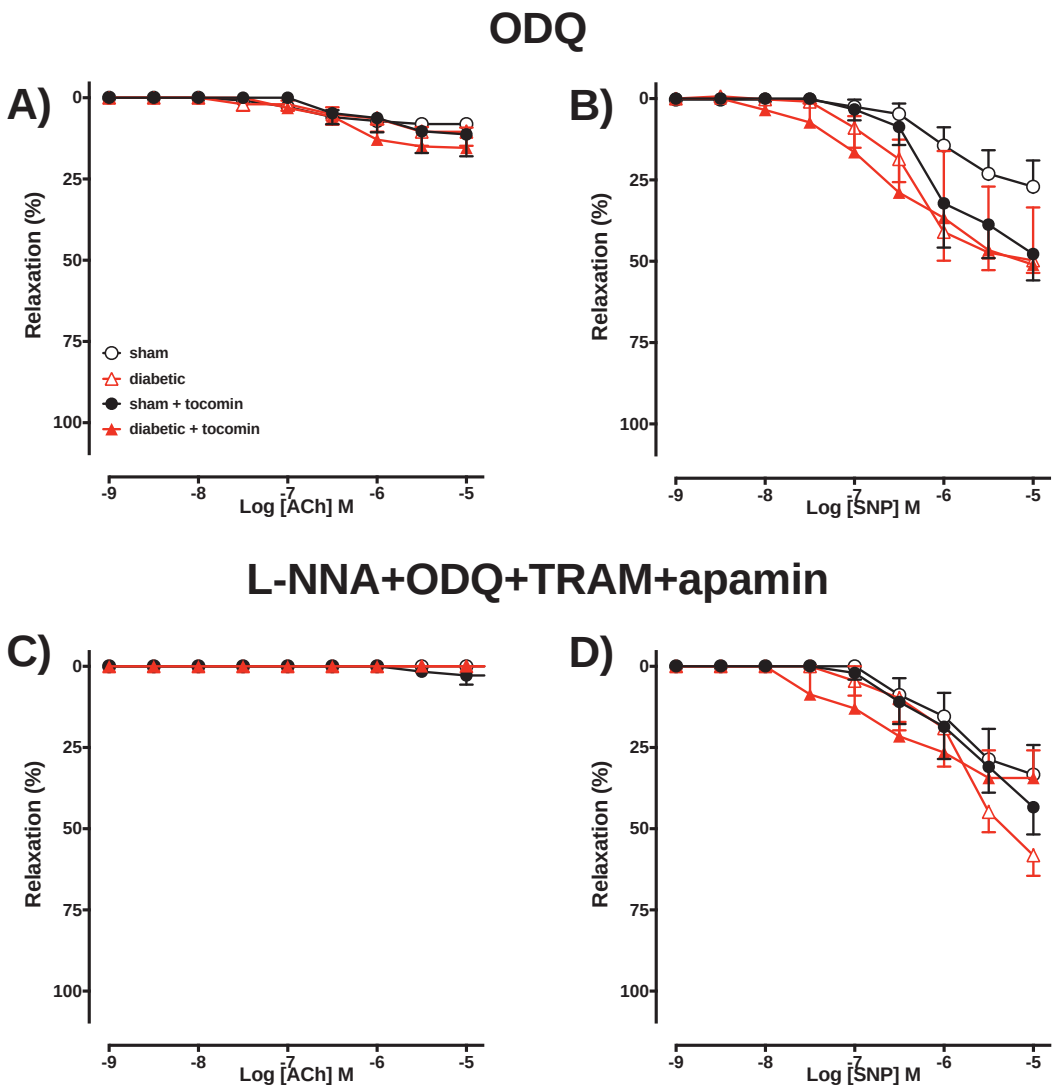


Figure 5.3 Cumulative concentration–response curves to ACh and SNP in endothelium-intact aortae isolated from sham, diabetic, and tocomin treated (sham + tocomin/diabetic + tocomin) rats in the presence of L-NNA + ODQ (A&B) and L-NNA + ODQ + Tram + apamin (C&D). Data is expressed as mean±SEM. $p<0.05$. Sidak's multiple comparison test. *n*=4-8. See Table 5.2 for values and statistical comparison.

5.3.4 The effect of diabetes and tocomin treatment on Nox2, eNOS, and modulatory proteins.

The effect of diabetes on the expression of Nox2 was significantly increased in aortae from diabetic in comparison to the sham rats (Figure 5.4). 4 week tocomin treatment significantly reduced Nox2 expression in aortae from diabetic rats. The total expression of the NO producing enzyme eNOS was significantly lower in aortae from diabetic in comparison to sham rats (Figure 5.5A). In addition, in the diabetic rat, aortae expression of calmodulin (CaM) and caveolin-1 was not decreased in comparison to the sham rats. The proportion of Akt that was phosphorylated was also not different in the diabetic rats in comparison to the sham (Figure 5.5B-D). Treatment with tocomin reversed the diet-induced changes in eNOS and Nox2 expression (Figure 5.5A). Treatment with tocomin did not affect expression of CaM and cav-1 neither the ratio of pAkt to Akt.

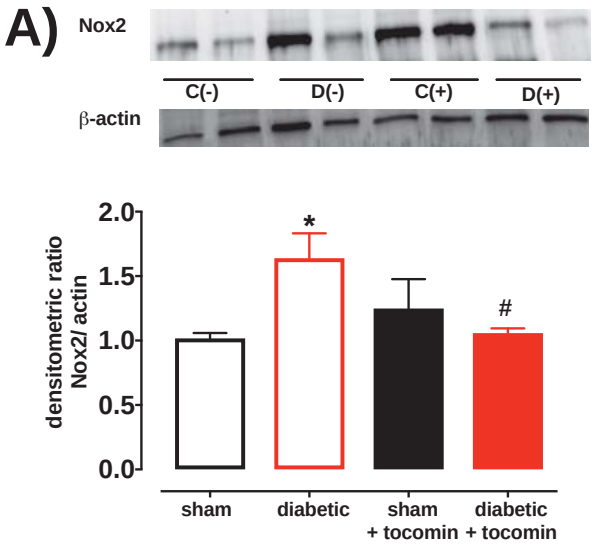


Figure 5.4 Protein expression of NADPH oxidase (Nox2) from isolated aortae from sham, diabetic and tocomin treated (sham + tocomin/diabetic + tocomin) rats. Representative blots are shown for each corresponding graph. *Significantly different to sham. #Significantly different to diabetic. Results are shown as means ± SEM. $p<0.05$. Two-way ANOVA Dunnett's multiple comparison test. n = 3-5 (pooled) experiments.

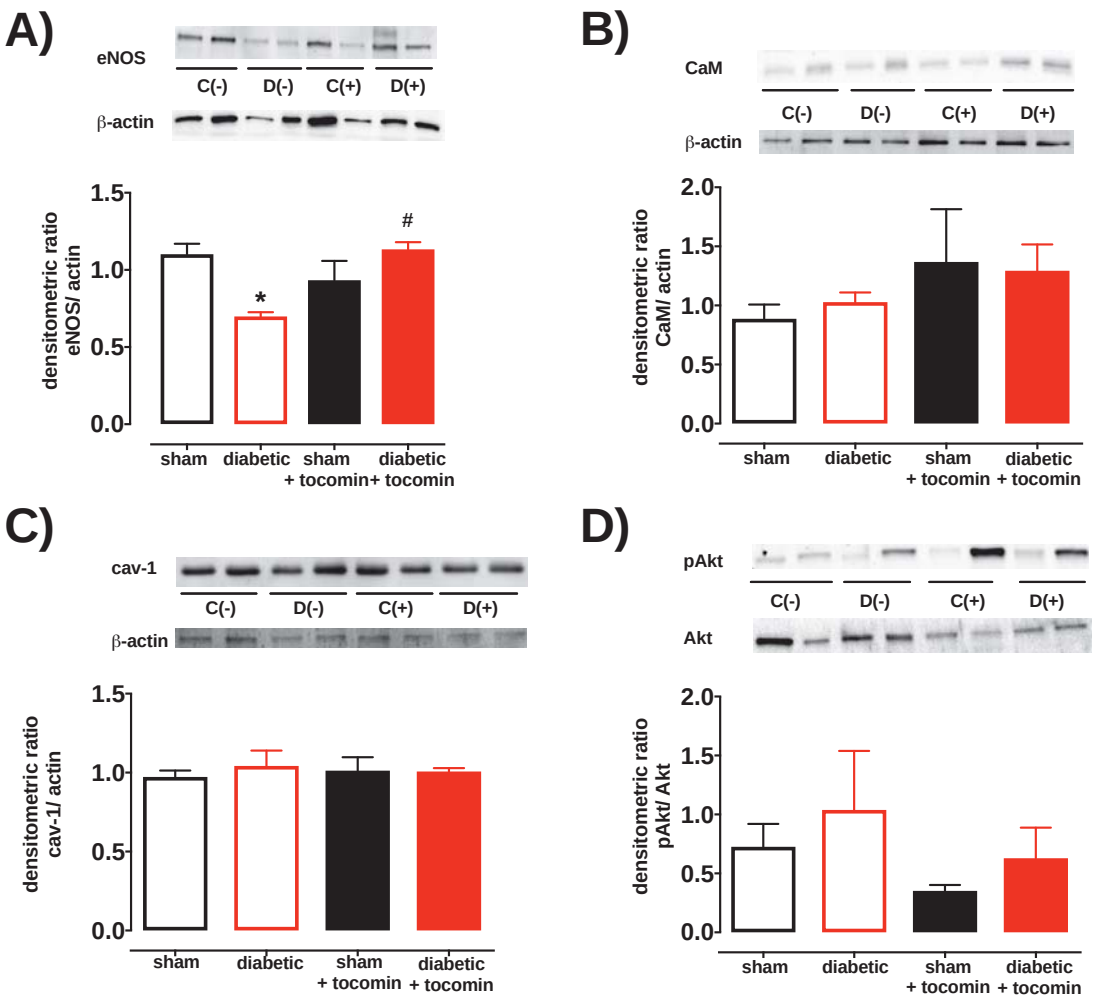


Figure 5.5 Protein expression of total eNOS (a), calmodulin-1 (B), caveolin-1 (C), and pAkt/Akt (D) from isolated aortae from sham, diabetic and tocomin treated (sham + tocomin/diabetic + tocomin) rats. Representative blots are shown for each corresponding graph. *Significantly different to sham. #Significantly different to diabetic. Results are shown as means ± SEM; $p<0.05$. Two-way ANOVA Dunnett's multiple comparisons test. n = 3-5 (pooled) experiments.

5.3.5 The effect of a high-fat WD and tocomin treatment on body weights, blood glucose and HbA1c.

The body weight of the western diet fed rats was significantly greater than that of the standard diet fed rats at the end of the experimental period. The final bodyweight of the tocomin treated western diet rats was not significantly different to the standard diet fed rats. The epididymal fat mass was significantly increased proportionate to final bodyweight in both of the western diet fed rats when compared to the standard diet fed rats. 4-week treatment tocomin had no effect on epididymal fat mass (Table 5.3).

The blood glucose and glycated hemoglobin (HbA1c) levels were not significantly different to the western diet fed rats. Blood glucose and glycated hemoglobin levels were not significantly affected by a western diet or 4-week tocomin treatment (Table 5.3).

Table 5.3: Mean body weight, blood glucose, HbA1c levels, and epididymal fat mass at the end of the experimental period for standard diet (SD) and western diet (WD) and tocomin (SD + tocomin/WD + tocomin) treated rats rats.

	n	SD	n	WD	n	SD + tocomin	n	WD + tocomin
Final Body Weight (g)	10	415 ± 10	8	458 ± 12*	10	421± 9	10	440± 8
Blood Glucose (mM)	9	8.4 ± 1	10	8.6 ± 0.9	9	8.8± 1	10	9.0± 1.3
HbA1c (%)	9	5.4 ± 0.3	10	5.2 ± 0.1	8	6.2± 0.3	10	5.9± 0.4
Epididymal fat (g)	10	8.5± 0.8	10	12.9 ± 1.2*	10	9.8± 1	10	12.4± 0.9*
Epididymal fat (% bodyweight)	10	2± 0.2	10	2.8 ± 0.2*	10	2.3± 0.2	10	2.8± 0.2*

*Significantly different to SD
Results are shown as mean±SEM.
Two-way ANOVA, $p<0.05$.

5.3.6 The effect of a high-fat WD and tocomin treatment on superoxide production in the rat aorta.

Basal superoxide production was significantly elevated in aortae from rats fed the WD in comparison to the SD rat aortae. The 4-week treatment with tocomin (40 mg/kg/day sc) significantly attenuated superoxide production by the aortae from a WD fed rat without affecting superoxide production by the SD rat aortae (Figure 5.6). Treatment with apocynin, a non-selective inhibitor of NADPH oxidase, decreased superoxide levels in the aorta from WD fed rats.

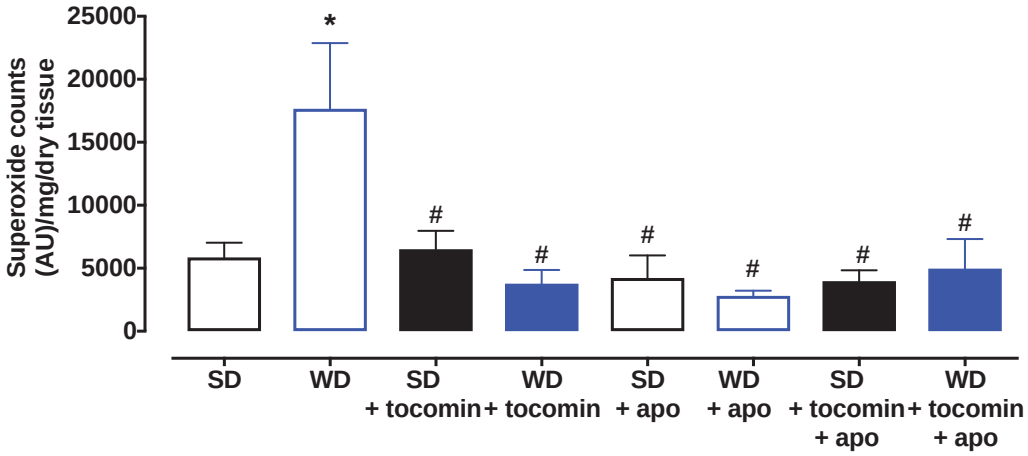


Figure 5.6 Superoxide generated in rat aorta in the presence of NADPH from SD, WD and tocomin treated (SD + tocomin/WD + tocomin) groups. *Significantly different to SD. #Significantly different to WD. Results are shown as mean±SEM. $p<0.05$. Two-way ANOVA Dunnet's multiple comparisons test. *n*=3-6.

5.3.7 The effect of a high-fat WD and tocomin on basal nitric oxide levels.

The contractile response of the aortae to the presence of the eNOS inhibitor L-NAME was significantly less in the aortae from WD rats compared to the SD rat aorta (Figure 5.7). The 4-week treat-ment of the WD rats with tocomin significantly improved the contractile response to L-NAME indicating increased basal NO synthesis.

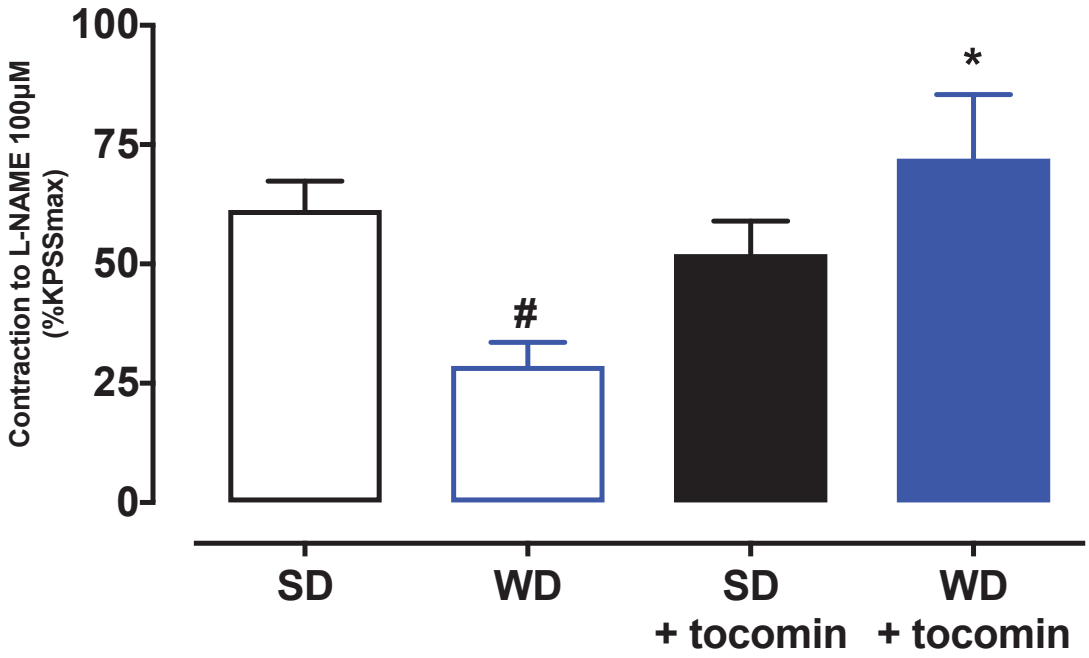


Figure 5.7 Response to L-NAME in the presence of KPSS from SD, WD and tocomin treated (SD + tocomin/WD + tocomin) groups. *Significantly different to SD. #Significantly different to WD. Results are shown as mean±SEM. $p<0.05$. Two-way ANOVA Dunnet's multiple comparisons test. *n*=3-8.

5.3.8 The effect of a high-fat WD and tocomin on endothelial function.

The sensitivity (pEC50), but not the maximum response, to the endothelium-dependent dilator ACh was significantly reduced in the aortae from WD compared to SD rats (Table 5.4; Figure 5.8A). Responses to SNP were not affected by a WD (Table 5.4; Figure 5.8B) indicating that a high-fat diet selectively impairs endothelium- dependent relaxation. The 4-week treatment of the WD rats with tocomin (40 mg/kg/day sc) significantly improved sensitivity to ACh in the aorta from WD-fed, but not SD-fed, rats. Tocomin treatment did not affect the endothelium-independent relaxation to SNP in any group.

In the presence of inhibitors of SK_{Ca} (apamin) and IK_{Ca} (TRAM- 34), ACh-induced relaxation is mediated by NO. The sensitivity to ACh was significantly decreased in the WD rat aorta compared to the SD aorta (Table 5.8; Figure 5.8C). Following 4-week treatment with tocomin, there was no change in ACh-induced relaxation in aortae from rats fed the SD, but the sensitivity to ACh was significantly increased in the WD rat aorta by the tocomin treatment. Tocomin treatment had no effect on the endothelium-independ-ent relaxant responses to SNP in any group (Table 5.4; Figure 5.8D). This suggests that tocomin treatment improves endothelial release of NO from the WD rat aorta rather than influencing sensitivity to either endogenous or exogenous NO.

In the presence of the eNOS inhibitor L-NAME alone and in combination with the sGC inhibitor ODQ, the maximum relaxation to ACh was significantly decreased in SD rat aortae (Table 5.4; Figure 5.8D), indicating the predominant contribution of NO to endothelium-dependent relaxation in this large artery. ACh-induced relaxation in the

presence of L-NAME, with or without ODQ, was not different in the aortae from WD rats compared to SD rats (Table 5.4; Figure 5.9A), and tocomin did not affect responses in either groups. These results suggest that in both the SD and WD rat aortae eNOS derived NO is the main cause of endothelium-dependent relaxation and 4-week treatment of WD rats with tocomin does not improve ACh sensitivity through an eNOS/sGC-independent mechanism. The presence of TRAM-34 and apamin, in addition to L-NAME and ODQ, did not further attenuate responses to ACh in any group (Table 5.4; Figure 5.9C) indicating an absence of contribution of calcium-activated potassium (K_{Ca}) channels to endothelium- dependent relaxation in this large artery.

L-NAME did not significantly affect responses to SNP (Figure 5.8F), but addition of ODQ did significantly reduce the sensitivity and maximum response to SNP in aortae from both SD and WD rats (Table 5.4; Figure 5.9B). Interestingly, in the presence of L-NAME and ODQ, SNP-induced relaxation was significantly greater in aortae from WD compared to SD rats (Table 5.4; Figure 5.9D). Tocomin did not affect responses to SNP under any of the tested conditions.

Table 5.4: The effect of 4-week tocomin treatment on ACh-induced endothelium-dependent and SNP-induced endothelium-independent relaxation of rat aortae fed a standard diet (SD) or high fat western diet (WD).

		ACh		SNP	
	n	**pEC_{50} (M)**	**R_{max} (%)**	**pEC_{50} (M)**	**R_{max} (%)**
Standard Diet (vehicle)					
Control	10	7.24±0.10	91±3	8.30-±0.17	95±3
Tram + apamin	10	7.24±0.15	85±5	7.99±0.26	99±2
L-NNA	7	6.47±0.27	24±5*	8.36±0.21	99±2
L-NNA + ODQ	9	6.82±0.23	8±3*	6.63±0.38*	46±12*
L-NNA + ODQ + tram + apamin	4	6.23±0.58	8±4*	5.85±0.29*	37±10*
Western Diet (vehicle)					
Control	8	6.85±0.12#	83±2	8.35±0.10	98±3
Tram + apamin	8	6.77±0.16#	84±2	7.62±0.21	100±3
L-NNA	7	6.70±0.16	26±8*	8.22±0.24	102±2
L-NNA + ODQ	7	6.30±0.11	17±2*	6.60±0.25*	67±4*
L-NNA + ODQ + tram + apamin	8	6.12±0.11	13±1*	6.00±0.24*	64±6*
SD + tocomin (40 mg/kg/day)					
Control	8	7.27±0.11	89±3	8.30±0.13	92±5
Tram + apamin	8	7.19±0.10	86±5	7.95±0.26	95±4
L-NNA	6	6.70±0.22	11±4*	8.40±0.26	98±3
L-NNA + ODQ	9	6.97±0.34	12±6*	6.19±0.64*	35±14*$
L-NNA + ODQ + tram + apamin	8	6.41±0.27	8±3*	4.70±0.9*	38±10*$
WD + tocomin (40mg/kg/day)					
Control	10	7.44±0.12$	89±2	8.35±0.10	98±3
Tram + apamin	9	7.27±0.10$	87±3	7.96±0.24	98±1
L-NNA	9	6.16±0.27	30±4*	8.30±0.13	101±4
L-NNA + ODQ	9	6.33±0.23	18±2*	6.45±0.33*	50±12*
L-NNA + ODQ + tram + apamin	9	6.50±0.22	7±2*	6.21±0.37*	63±9*

*Significantly different to control
#Significantly different to SD of same treatment group
$Significantly different to WD of same treatment group
Results are shown as mean±SEM.
Two-way ANOVA, $p<0.05$. Sidaks multiple comparison test.

Table illustrates the pEC_{50} and Rmax values for ACh in aortic rings from standard diet (SD), western diet (WD) and tocomin treated (SD+tocomin/WD+tocomin) groups. The effect of various chemical inhibitors is shown. n=no. of experiments.

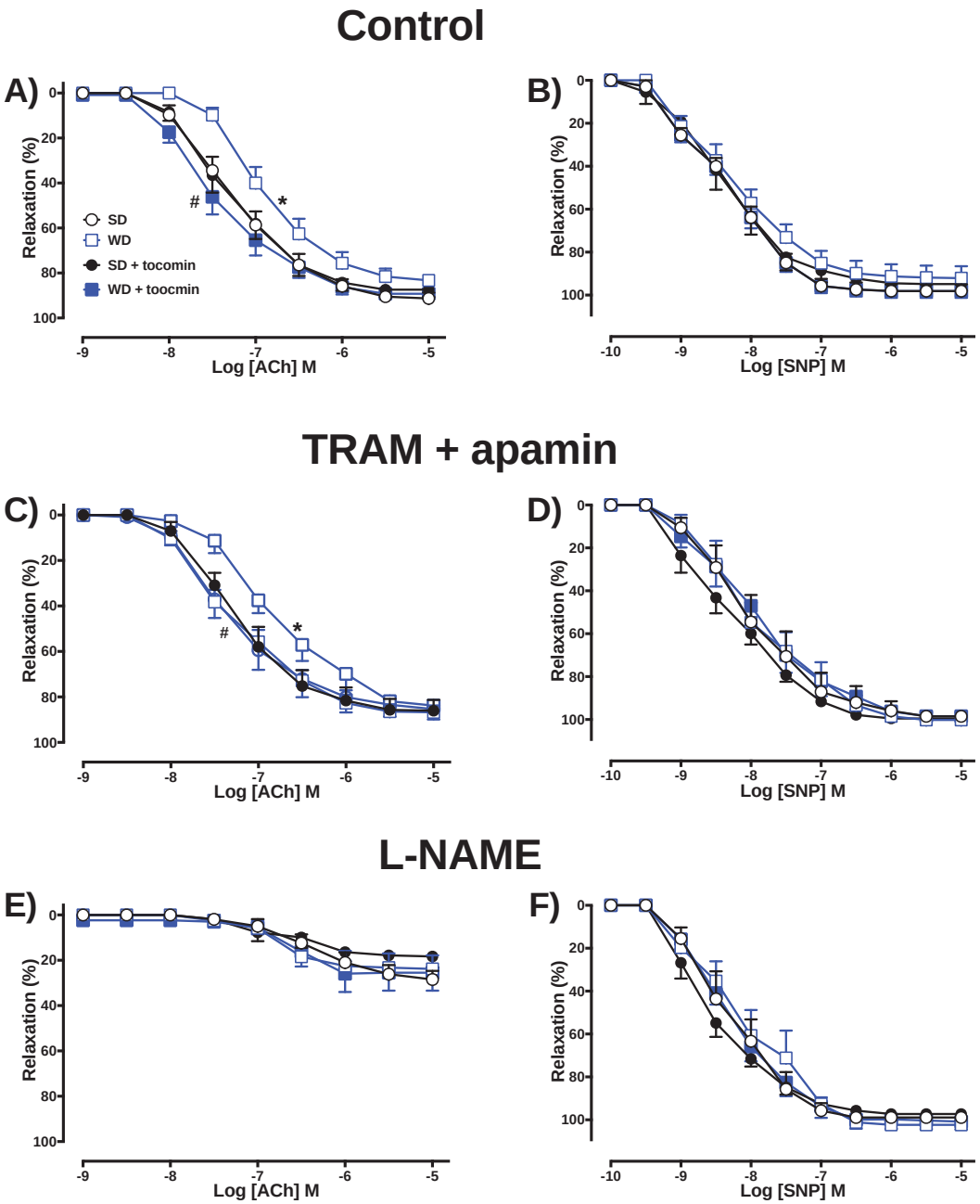


Figure 5.8 Cumulative concentration–response curves to ACh and SNP in endothelium-intact aortae isolated from standard diet (SD), western diet (WD), and tocomin treated (SD + tocomin/WD + tocomin) rats in control (A&B), or in the presence of Tram + apamin (C&D), and L-NAME (E&F). *pEC_{50} Significantly different to SD. $^{\#}pEC_{50}$ Significantly different to WD. Data is expressed as mean±SEM. $p<0.05$. Sidak's multiple comparison test. *n*=4-10. See Table 5.4 for values and statistical comparison.

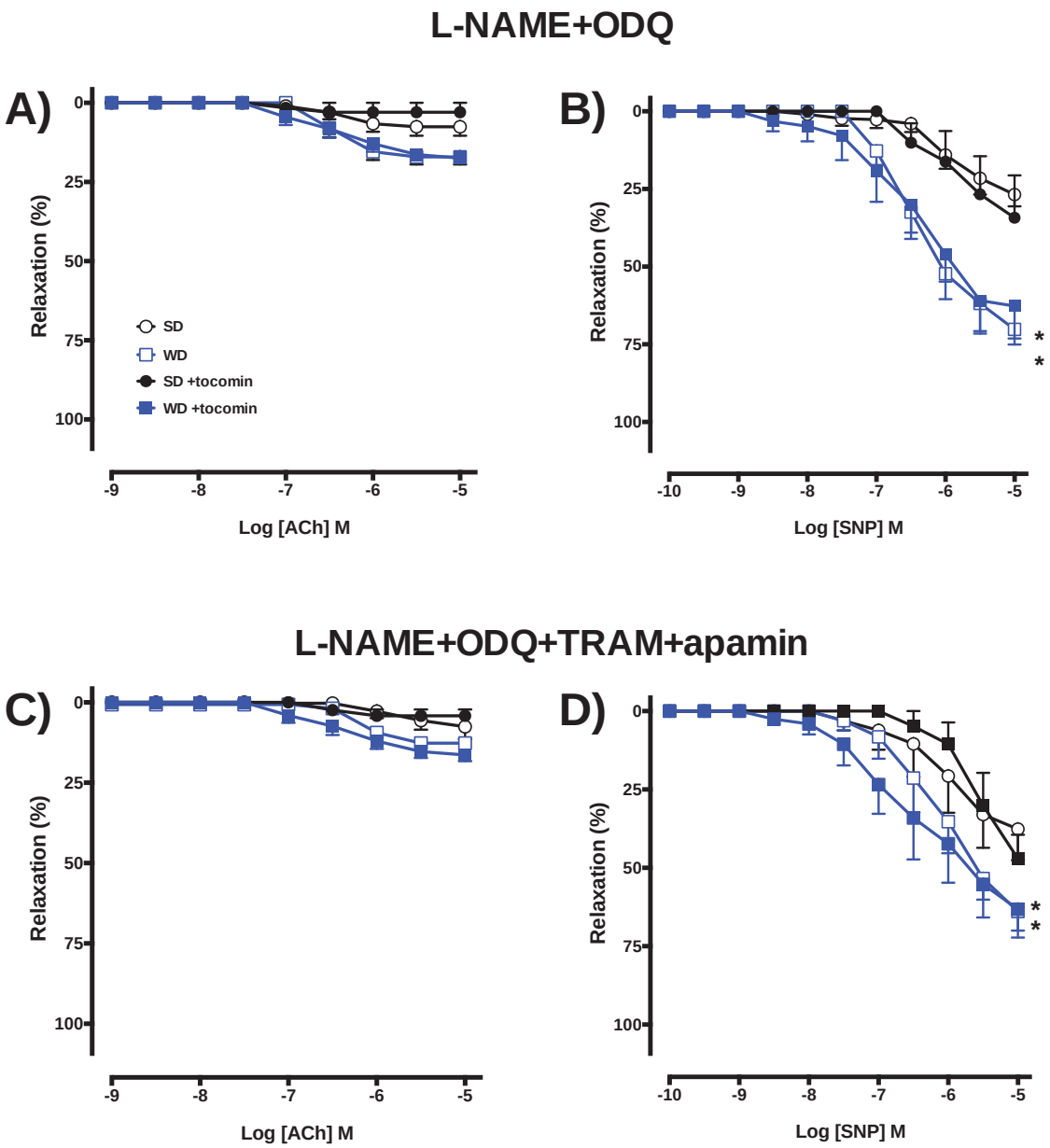


Figure 5.9 Cumulative concentration–response curves to ACh and SNP in endothelium-intact aortae isolated from SD, WD, and tocomin-treated (SD + tocomin/WD + tocomin) rats in the presence of L-NAME + ODQ (A&B) and L-NAME + ODQ + Tram + apamin (C&D). *R_{max} significantly different to SD. Data is expressed as mean±SEM. $p<0.05$. Sidak's multiple comparison test. *n*=4-8. See Table 5.4 for values and statistical comparison.

5.3.9 The effect of a high-fat WD and tocomin treatment on Nox2, eNOS, and modulatory proteins.

The effect of a high fat WD on the expression of superoxide producing enzyme Nox2 was significantly increased in aortae from WD in comparison to the SD rats (Figure 5.10). Tocomin treatment did not affect Nox2 in aortae from SD rats but significantly reduced its expression in aortae from WD rats. The total expression of the NO producing enzyme eNOS was significantly lower in aortae from WD in comparison to SD rats (Figure 5.11A). In addition, in WD rat, aortae expression of calmodulin (CaM) was decreased, and caveolin-1 was increased in comparison to SD rats. The WD also decreased the proportion of Akt that was phosphorylated (Figure 5.11D). Treatment with tocomin reversed the diet-induced changes in eNOS, caveolin-1, CaM, and the phosphorylation of Akt (Figure 5.11D).

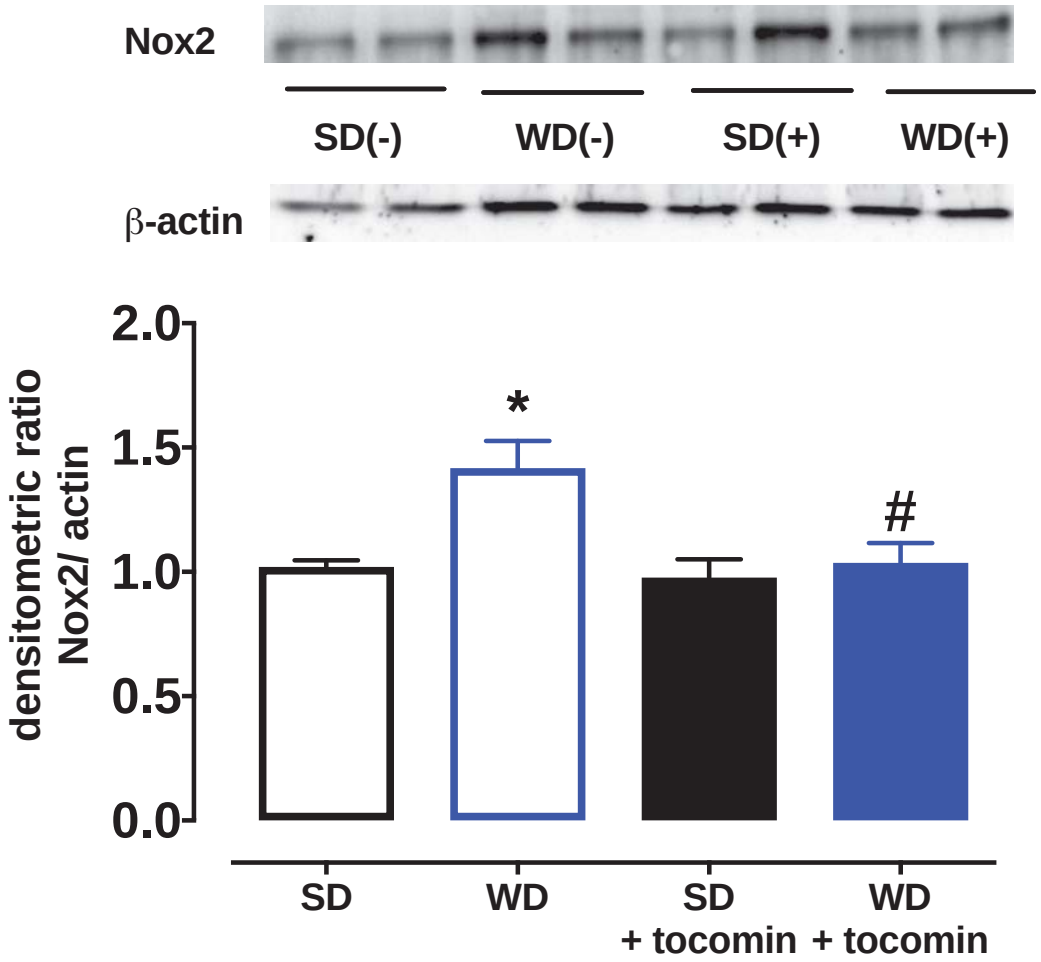


Figure 5.10 Protein expression of NADPH oxidase (Nox-2) from isolated aortae from SD, WD and tocomin treated (SD+tocomin/WD+tocomin) rats. Representative blots are shown for each corresponding graph. *Significantly different to SD. #Significantly different to WD. Results are shown as means ±SEM. p <0.05. 2-way ANOVA Dunnet's Results are shown as means ± SEM. p<0.05. Two-way ANOVA Dunnett's multiple test. *n* = 6 experiments.

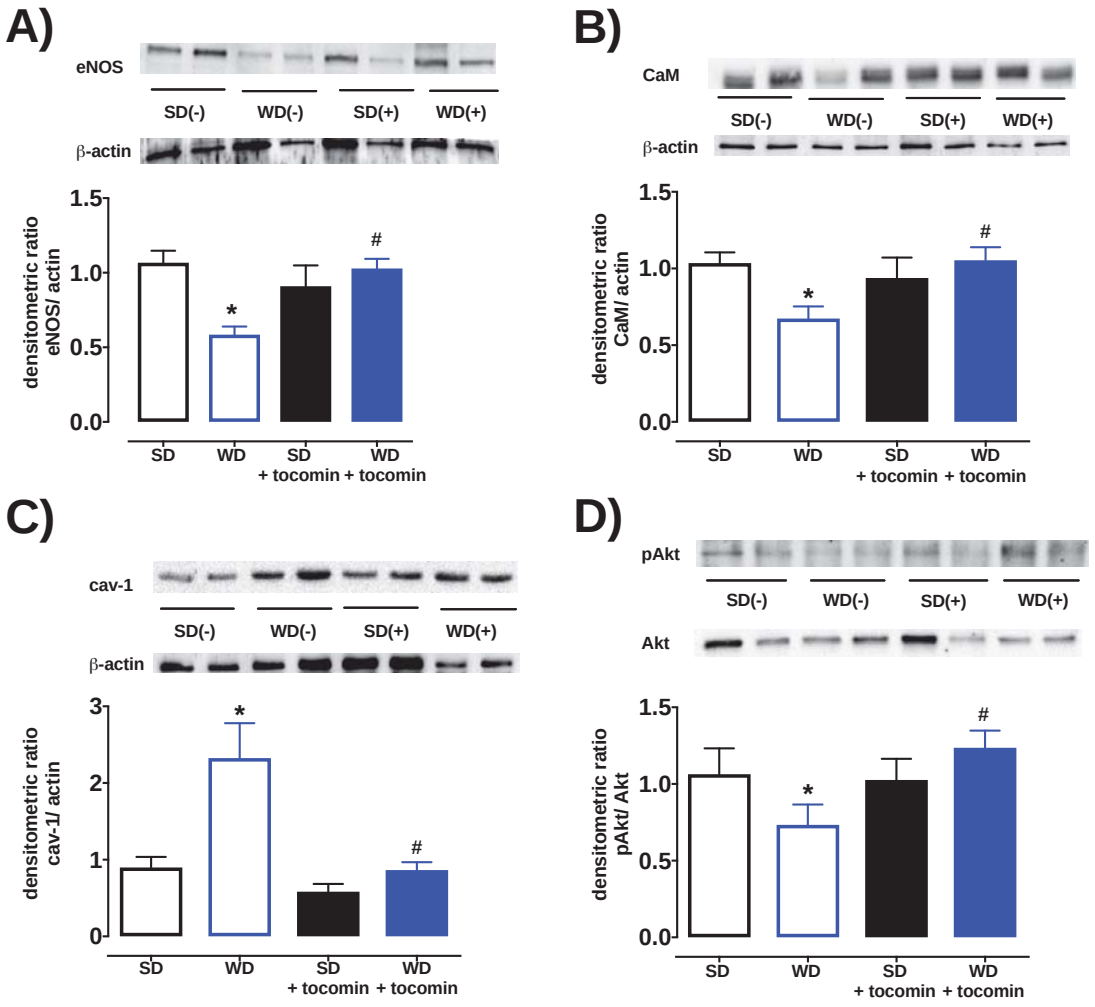


Figure 5.11 Protein expression of total eNOS (a), calmodulin-1 (B), caveolin-1 (c), and pAkt/Akt (D) from isolated aortae from SD, WD, and tocomin treated (SD + tocomin/WD + tocomin) rats. Representative blots are shown for each corresponding graph. *Significantly different to SD. #Significantly different to WD. Results are shown as means ± SEM. $p<0.05$. Two-way ANOVA Dunnett's multiple comparisons test. n = 6 experiments.

5.4 Discussion

There is ample of literature that demonstrates the contribution of oxidative stress to the impairment of endothelium-dependent relaxation in the vasculature. This study has demonstrated that the tocotrienol rich tocomin acutely reduces the levels of oxidative stress and improves endothelium-dependent relaxation in aortae from type 1 diabetic and western diet fed rats.

This study demonstrates that during diabetes, an impairment of endothelium-dependent relaxation that is associated with an increased expression of the NADPH oxidase Nox2 subunit in the aorta and an increase in the vascular generation of O_2^-. The increase in vascular oxidative stress was accompanied by an impairment of NO mediated ACh-induced relaxation. A decreased expression of eNOS is likely to contribute to the impaired NO-mediated relaxation. Diabetes did not affect calmodulin and cav-1 expression, it also did not affect the ratio of phosphorylated Akt to Akt in the aortae. Tocotrienol rich tocomin was able to improve NO mediated relaxation in the diabetic rat aortae by increasing NO bioavailability, most likely due to an antioxidant action, a property of tocomin that we previously demonstrated in isolated vascular tissue (Chapters 3 and 4).

The results of this study also demonstrate that rats fed a high-fat, "western" diet exhibit an impairment of endothelium-dependent relaxation that is also associated with an increased expression of the NADPH oxidase Nox2 subunit in the aorta and an increase in basal levels of superoxide. The increase in vascular oxidative stress was accompanied by a decrease in basal NO release in the WD rat aorta and an impairment of the contribution of NO to ACh-induced relaxation. A decreased expression of eNOS,

calmodulin, and phosphorylated Akt and an increase in caveolin-1 are likely to contribute to the impaired NO-mediated relaxation.

5.4.1 The effect of diabetes and tocomin treatment on bodyweight blood glucose levels and HbA1c.

Type 1 diabetes caused a significant increase in BGL and HbA1c levels and a significant decrease in bodyweight. However, treating diabetic rats with tocomin did not affect the blood glucose or HbA1c levels nor did it increase bodyweight.

5.4.2 The effect of diabetes and tocomin treatment on oxidative stress and endothelial function during diabetes.

There was a significant increase in O_2^- generation in arteries from diabetic rats, but not in the sham rat aortae. This was accompanied with an increase in Nox2 expression, once again only in the diabetic rat aortae. Four week tocomin treatment was able to significantly attenuate O_2^- generation and reduce Nox 2 expression in the diabetic rat aortae. The results from this study are consistent with the findings of Chapters 3 and 4 that demonstrated tocomin acutely reduced O_2^- production in isolated aortae from diabetic rats and in the presence of oxidative stress. A possible mechanism through which tocomin treatment decreases Nox2 expression in the diabetic rat aortae could be through decreasing PKC activation. PKC is a family of proteins that have many functions that regulate cell growth, PKC are also a positive regulator of Nox2 activity by activating the enzyme (Seshiah et al., 2002). PKC-induced Nox2 activation is known to be involved in the pathophysiology of CAD and atherosclerosis (Inoguchi et al., 2000). A study conducted by Clement et al., (1997) demonstrated that the treatment of

rat aortic smooth muscle cells with α-tocopherol could inhibit PKC activation. This could subsequently lead to decreased Nox2 activation and expression therefore decreasing oxidative stress. This α-tocopherol-induced inhibition of PKC activation was believed not to be connected to the antioxidant function of α-tocopherol indicating that α-tocopherol induced PKC inhibition is achieved by inhibition at a cellular level rather than a direct antioxidant effect of α-tocopherol (Carew et al., 1987, Esterbauer et al., 1992). We hypothesize that the tocomin-induced decrease in Nox2 expression that has been observed in this study could be explained by a similar mechanism, i.e; tocomin that is rich in tocotrienols, inhibits lipid peroxidation through its antioxidant function. Inhibition of lipid peroxidation leads to decrease activity of the DAG kinase enzyme that is a stimulator of PKC. By reducing DAG kinase dependent PKC activation tocomin has a direct effect on decreasing PKC activation and subsequent Nox2 activation and expression. This phenomenon has been demonstrated by Lee et al., (1996) that demonstrated vitamin E (α-tocopherol) and its ability to prevent hyperglycemia-induced activation of the DAG-PKC pathway in vascular smooth muscle cell via an decrease in DAG kinase activity. As tocomin shares structural similarity and functionality as α-tocopherol, tocomin may also be acting through a similar mechanism. Confirmation of this possibility however would require further investigation.

Diabetes also caused a significant impairment of endothelium-dependent relaxation as was observed in the Chapter 4. Four-week treatment of the diabetic rats with tocomin significantly improved vascular relaxation. Neither diabetes nor tocomin treatment had any effect on responses to SNP in aortae from diabetic. Thus tocomin, which we have previously reported to acutely enhance endothelium-dependent relaxation in diabetic

and isolated vascular tissue exposed to oxidative stress (Chapters 3 and 4), is also able to improve endothelial function when administered *in vivo*.

5.4.3 The effect of diabetes and tocomin treatment on NO-mediated relaxation, eNOS and its regulatory proteins.

Following investigation of the effect of diabetes and tocomin treatment on endothelium-dependent relaxation we wanted to investigate whether the NO-mediated component of that response was influenced by diabetes and/or by tocomin treatment by examining responses to ACh in the presence of TRAM-34 plus apamin to eliminate the EDH-type component of relaxation. In the presence of SK_{Ca} and IK_{Ca} channel blockers TRAM-34 and apamin, the ACh-induced relaxation was significantly impaired in aortae in the diabetic rats compared to sham rats indicating that diabetes impaired NO-mediated relaxation. This has been demonstrated previously (Joshi and Woodman, 2012, Leo et al., 2012). NO mediated relaxation was significantly improved in the diabetic rat indicating that 4-week tocomin improves NO bioavailability.

The expression of eNOS was significantly reduced during diabetes. However, the expression of CaM, caveolin-1, pAkt and Akt was not affected by diabetes or 4-week tocomin treatment indicating that pAkt/Akt-or CaM induced eNOS activation and cav-1-induced eNOS inhibition play no role in the endothelial dysfunction that was seen in this study. The effect of diabetes on eNOS uncoupling has been previously demonstrated (Leo et al., 2011a) and whether tocomin improves eNOS coupling in the diabetic rat aortae requires further investigation. Treatment with tocomin significantly increased NO-mediated relaxation in diabetic aortae but had no effect on relaxation of sham vessels. Tocomin treatment caused several effects that might have contributed to

the increased NO activity in the diabetic aortae. First, the decrease in Nox2 would contribute to the decrease in vascular superoxide generation reducing the inactivation of NO and formation of $ONOO^-$. Furthermore, tocomin treatment increased expression of eNOS in comparison to the sham rats.

Tocomin treatment did not affect the response to SNP under any conditions. The ability of tocomin to enhance responses only to endogenous NO supports the importance of the treatment on improvement of the endogenous generation of NO through the increased expression of eNOS.

5.4.4 The effect of diabetes and tocomin treatment on EDH-type relaxation.

To investigate how diabetes affects EDH-type relaxation and whether tocomin improves endothelium-dependent relaxation through an improvement in EDH-type relaxation we observed ACh-induced endothelium-dependent relaxation in the presence of eNOS inhibitor L-NNA alone, L-NNA plus ODQ and L-NNA. In the presence of L-NNA alone endothelium-dependent was significantly reduced in both sham and diabetic rat aortae. However interestingly, in the presence of L-NNA alone the maximum response was significantly larger (Figure 5.2E) in the diabetic aortae in comparison to the sham rat aortae. This indicates that EDH-type relaxation helps preserve endothelium-dependent relaxation in the diabetic aortae. Neither diabetes nor tocomin treatment had any effect to the responses to SNP in the presence of L-NNA.

In the presence of L-NNA plus ODQ and all of the inhibitors in combination both endothelium-dependent and independent relaxation was significantly reduced

demonstrating that NO dependent sGC activation is necessary for relaxation in the aortae. Responses to SNP in the presence of L-NNA and ODQ were not affected by diabetes nor tocomin treatment.

5.4.5 The effect of high-fat WD and tocomin treatment on bodyweight, blood glucose levels and HbA1c.

The high-fat WD did not cause any increase in blood glucose levels or HbA1c at the end of the study, but the WD group had a significantly higher body weight and epididymal fat mass at the end of the feeding period (Table 5.3) similar to observations previously made when using the same diet (Kosari et al., 2012). Epididymal fat pad mass provides an established indication of obesity (Mathai et al., 2008).

5.4.6 The effect of a high-fat WD and tocomin treatment on oxidative stress and endothelium-dependent relaxation.

The WD caused a significant increase in the superoxide generated by the aortae and this correlated with an increase in the expression of the NADPH oxidase subunit Nox2. Others have reported that diets high in fats and sugars cause oxidative stress in the cardiovascular system (Chapter 4) (Malakul et al., 2008, Roberts et al., 2005). Our observation that there was an increased expression of Nox2 that might contribute to the generation of vascular oxidative stress is similar to a previous report that a high-fat, high-sucrose diet increased vascular Nox2, but not Nox4, expression in mice (Qin et al., 2014). Four week treatment of the WD rats with tocomin significantly reduced Nox2 expression similarly to the tocomin treated diabetic rats. The mechanism for decreased Nox2 expression observed in the tocomin treated WD rat aortae could be similar to the diabetic rat aortae where tocomin acts at a cellular level to reduce PKC activated Nox2 O_2^- production that subsequently reduces oxidative stress in the

vasculature thus improving NO bioavailability and endothelial dysfunction. However, this possibility requires further investigation.

The WD-induced increase in oxidative stress was accompanied by a decreased contractile response to the NOS inhibitor L-NAME and an impaired response to the endothelium-dependent relaxant ACh without any effect on the NO donor SNP. These observations are indicative of a selective impairment of endothelial function in the rat aortae as has been previously reported (Jenkins et al., 2016, Wang et al., 2016), when there is an increase in oxidative stress including in response to consumption of a high-fat diet (Kshirsagar et al., 2015, Roberts et al., 2005).

5.4.7 The effect of a high-fat WD and tocomin on endothelial function.

Treating WD rats with tocomin did not affect the blood glucose or HbA1c levels nor did it decrease bodyweight or epididymal fat mass. However, there was a significant decrease of superoxide generation in arteries from WD, but not SD, fed rats and a decrease in the Nox2 expression, once again only in the WD fed rat aortae. Tocomin treatment did not affect responses to ACh or SNP in aortae from SD fed rats but did increase the sensitivity to ACh in the WD fed rat aortae. Thus tocomin, which we have previously reported to acutely enhance endothelium-dependent relaxation in isolated vascular tissue exposed to oxidative stress (Chapter 3), is also able to improve endothelial function when administered *in vitro*.

5.4.8 The effect of a high-fat WD and tocomin treatment on NO-mediated relaxation, the expression of eNOS and its regulatory proteins.

We next investigated whether the NO-mediated component of the endothelium-dependent relaxation was influenced by the WD and/or by tocomin treatment by examining responses to ACh in the presence of the K_{Ca} channel blockers TRAM-34 plus apamin to eliminate the EDH-type component of relaxation. In the presence of TRAM-34 and apamin, the ACh-induced relaxation was significantly impaired in aortae from WD rats compared to SD rats indicating that the high-fat diet impaired NO-mediated relaxation. This impairment of responses to stimulated release of NO was consistent with the observation that the basal release of NO was also impaired in WD vessels, demonstrated by a decreased contractile response to NOS inhibition. A number of factors may have contributed to the WD-induced impairment of NO release. The expression of eNOS was significantly reduced by consumption of the high-fat diet. Further, the expression of caveolin-1, which inactivates eNOS, was increased, whereas calmodulin and pAkt expression was decreased. Calmodulin and pAkt both act to promote eNOS activity (Fleming, 2010, Takahashi and Mendelsohn, 2003). eNOS activity may also be influenced by the phosphorylation of Ser1179/1177 and Thr495, among other key sites (Siragusa and Fleming, 2016), but eNOS phosphorylation was not investigated in this study. The changes in modulatory protein expression are similar to changes observed under other circumstances of increased oxidative stress, such as diabetes (Leo et al., 2011a, Leo et al., 2010a). It is also well established that oxidative stress may also cause uncoupling of eNOS to promote synthesis of superoxide rather than NO (Siragusa and Fleming, 2016). We have previously reported that diabetes causes eNOS uncoupling (Leo et al., 2011a) and, while we did not explore whether a high-fat diet has a similar effect in this study, obese Zucker rats are reported to have an

elevated level of uncoupled eNOS (Munoz et al., 2015). Treatment with tocomin significantly increased NO-mediated relaxation in WD aortae but had no effect on relaxation of SD vessels. Tocomin treatment caused several effects that might have contributed to the increased NO activity in the WD aortae. First, the decrease in Nox2 would contribute to the decrease in vascular superoxide generation reducing the inactivation of NO and formation of $ONOO^-$.

Furthermore, tocomin treatment increased expression of eNOS and calmodulin and increased phosphorylation of Akt to return levels in WD rats to be similar to those seen in SD rats. There was also a decrease in caveolin-1. Together, these actions are consistent with an increased capacity to synthesize NO reflected as both an improved basal release of NO, demonstrated by the contractile response to a NOS inhibitor, and enhanced ACh-induced relaxation reflecting stimulated NO release. An interesting observation was that SNP, an endothelium- independent NO donor, caused a significantly greater relaxation in WD versus SD aortae when NOS and sGC were inhibited. This is a surprising observation and there appear to be no previous reports of studies where responses to a NO donor were examined under similar conditions. The data suggest that WD may increase the activity of sGC or impair the inhibitory capacity of ODQ. Further investigation would be required to investigate these possibilities. Tocomin treatment did not affect the response to SNP under any conditions. The ability of tocomin to enhance responses only to endogenous NO supports the importance of the treatment on improvement of the endogenous generation of NO through the increased expression of eNOS, calmodulin, and pAkt and the decreased expression of caveolin-1.

5.4.9 The effect of a high-fat WD and tocomin on EDH-type relaxation.

To investigate whether the non-NO, EDH-type component of the endothelium-dependent relaxation was influenced by the WD and/or by tocomin treatment, we examined responses to ACh in the presence of the NOS inhibitor L-NAME and the sGC inhibitor ODQ. In this large artery from SD-fed rats, only a small response to ACh remained in the presence of L-NAME, and the addition of ODQ virtually abolished the remnant relaxation indicating that normally there was little if any role for EDH-type relaxation. Responses in aortae from WD rats were the same as in SD rats, and tocomin treatment did not change responses in any group indicating that there was no influence of the treatment on EDH- type relaxation. We have previously found that an antioxidant can improve NO but not EDH-type relaxation even in small arteries where non-NO mediators make a more marked contribution to relaxation than in the large artery used in this study (Takahashi and Mendelsohn, 2003). The small relaxation remaining in the presence of L-NAME, ODQ, TRAM, and apamin is most likely mediated by prostacyclin.

5.5. Conclusion

There are many animal and human studies that demonstrate the link between ROS and the development of cardiovascular disease (Ritchie et al., 2017) including in diseases associated with diabetes and obesity (Roberts et al., 2005, Stump et al., 2005). There has also been extensive research on the effect of antioxidants including vitamin E (predominantly α-tocopherol) in the prevention and treatment of cardiovascular disease, but despite many positive results in animal studies, they have not translated the same results in humans or have had mixed outcomes (Saremi and Arora, 2010). There is

growing evidence that tocotrienols possess more potent antioxidant activity in comparison to tocopherols (Serbinova et al., 1991). Further, we demonstrated in Chapter 3 that the combination of tocotrienols with α-tocopherol is more effective at preserving endothelial function in the presence of oxidative stress than either α-tocopherol alone or tocotrienols alone in combination (Chapter 3).

The outcome of the present study, where a tocotrienol-rich extract that includes some tocopherol preserves endothelial function in the presence of diabetes and obesity-induced oxidative stress, suggests further investigation of tocomin as a potential therapeutic agent is warranted. We have demonstrated that the *in vivo* treatment of diabetic and rats fed a high-fat "western" diet with the tocotrienol-rich extract of palm oil; tocomin, increases NO activity to improve endothelium-dependent relaxation in aortae from. Tocomin did not affect diabetes induced weight-loss and diet-induced weight gain or increase in epididymal fat but did attenuate the vascular oxidative stress. Our previous study demonstrated that tocomin, which contains a high proportion of tocotrienols (48%) with some α-tocopherol (11%), is able to acutely reduce oxidative stress to improve endothelium-dependent relaxation *in vitro* (Chapters 3 and 4). An additional beneficial action in this study to reduce vascular oxidative stress was a decreased expression of the vascular NADPH oxidase subunit Nox2 in both diabetic and WD rat aortae. Increased Nox2 expression is known to impact vascular function. This was demonstrated by Violi et al., (2009) where people with heredity Nox2 deficiency had enhanced endothelium-dependent relaxation. Another study conducted by Wang et al., (2016) demonstrated the antioxidant geraniol improves endothelium-dependent relaxation and reduces Nox2 expression in the aortae of high fat diet fed mice by down regulating Nox2 expression. Our results suggest that the antioxidant

activity of tocomin at scavenging O_2^- and reducing oxidative stress in the vasculature has a down stream effect on Nox2 activity that is possibly due to tocomin decreasing PKC- induced Nox2 activation at a cellular level, leading to a decrease in Nox2 activation and subsequent expression in the diabetic and obese aortae. However, this would require further investigation.

A further positive outcome of tocomin treatment in the obese rats was an increased expression of eNOS in the diabetic and WD aortae where in the WD aortae tocomin increased eNOS activity by promoting proteins calmodulin and pAkt. Further, there was also a decreased expression of the inhibitory protein caveolin-1. Interestingly neither diabetes or tocomin did not affect phosphorylated Akt, Akt, calmodulin or caveolin-1 expression. The beneficial actions of tocomin in this model of diabetes and diet-induced model of obesity suggest that it may have potential to be used as a therapeutic agent to prevent vascular disease in diabetes and obesity.

Chapter 6

General Discussion & Conclusion

CHAPTER 6: GENERAL DISCUSSION & CONCLUSION

There is a substantial body of literature that demonstrates the contribution of oxidative stress to the impairment of endothelium-dependent relaxation in the vasculature. This thesis has demonstrated that diabetes and obesity causes endothelial dysfunction in the aorta from diabetic and obese rats resulting from increased oxidative stress in the vasculature. We have also demonstrated that the tocotrienol rich tocomin is up to 100 times more potent than α-tocopherol at improving endothelial function acutely in the presence of oxidative stress and chronic animal models of diabetes and obesity. This thesis has demonstrated that α-tocopherol is a necessary component of tocomin and tocotrienols are not as effective as an antioxidant in the absence of α-tocopherol. This thesis also demonstrated that 4 week treatment of diabetic and obese rats with tocomin (40 mg/kg/day s.c.) reduces levels of oxidative stress and improves endothelium-dependent relaxation.

6.1 The effect of acute α-tocopherol, tocomin and tocotrienol isomer exposure in rat aortae with pyrogallol-induced oxidative stress.

The first study (Aims 1&2) investigated the effect of acutely exposing rat aortae to α-tocopherol, tocotrienol isomers, various combinations of tocotrienol isomers, tocotrienol rich tocomin and a mixture containing the same components as tocomin in the rat aortae of pyrogallol/NADPH-induced oxidative stress. We also studied the O_2^- scavenging capacity of the above-mentioned molecules in a tissue free system using hypoxanthine and xanthine oxidase as a O_2^- generator. The study demonstrated that that

α-tocopherol, tocomin, tocotrienol isomers individually and in combination were able to scavenge superoxide generated from hypoxanthine/xanthine oxidase (Hypothesis 1). However, tocomin was able to scavenge superoxide at a concentration 100 times lower than that of α-tocopherol. These findings are consistent with findings from Serbinova, 1991 which was one of the first studies that demonstrated α-tocotrienol to be 40-60 times more potent as an antioxidant in comparison to α-tocopherol (Serbinova et al. 1991).

It has been suggested that tocotrienols may have superior antioxidant activity to tocopherols (Peh et al., 2016, Singh et al., 2013b), and we did find that to be true when superoxide is generated by hypoxanthine/xanthine oxidase *in vitro* (Chapter 3). It was therefore surprising when observing the effect of the same molecules on endothelium-dependent relaxation in the presence of pyrogallol in isolated rat aortae α, *δ* and *γ*-tocotrienols and various combinations in the absence of *α*-tocopherol were largely ineffective in improving NO mediated, endothelium-dependent relaxation in the presence of oxidative stress. However, tocotrienol rich tocomin, that has a minor component of *α*-tocopherol, was found to be the most effective compound tested. We also demonstrated that tocomin was up to 100 times more effective than α-tocopherol at improving endothelium dependent relaxation in the presence of pyrogallol. Also the efficacy of tocomin could be replicated by the presence of *α*-tocopherol with *α*, *δ* and *γ*-tocotrienols but not by the combined presence of the 3 tocotrienols alone. Thus the combination of tocotrienol isomers and tocopherol may prove to be an effective approach to the preservation of endothelial function where there is disease-induced oxidative stress such as in diabetes and hypertension.

This study also demonstrated that the tocotrienol isomers were less effective at scavenging superoxide radicals produced by vascular tissue in the presence of NADPH in comparison to those generated by hypoxanthine/xanthine oxidase. α-Tocopherol was less effective than the tocotrienol isomers at similar concentrations when superoxide was generated by hypoxanthine/xanthine oxidase but more effective against superoxide generated by vascular tissue. Tocomin was effective in both assays at 100 fold lower concentrations than α-tocopherol. Surprisingly tocomin was the most effective compound at improving endothelium-dependent relaxation and this effect could be replicated by a mixture of α-tocopherol and α, δ and γ-tocotrienols in all of the assays, suggesting that the tocotrienol isomers provide more effective vasoprotection when acting together in combination with α-tocopherol. This indicates that α-tocopherol possesses non antioxidant properties, which are beyond the scope of this thesis, but has been demonstrated in other studies (Kannappan et al., 2012, Ahsan et al., 2014).

6.2 The effect of acute α-tocopherol and tocomin exposure in rat aortae animal models of diabetes and obesity.

After demonstrating the antioxidant activity of α-tocopherol and tocomin and their ability to improve endothelium-dependent relaxation in pyrogallol induced oxidative stress, the next study (Aim 3) compared the effect of diabetes and a high-fat diet rats western diet on endothelium-dependent relaxation in isolated aortae and whether acute exposure of α-tocopherol and tocomin can improve vascular relaxation.

Diabetic rats had increased BGL and HbA1c levels in contrast to the high-fat western diet rats that in the absence of high BG and HbA1c levels developed obesity that was

seen as a significant increase in epididymal fat in comparison to the sham rats. O_2^- production was significantly increased in both the diabetic and WD rat aortae that was associated with increased Nox2 expression and acute treatment of the aortae with tocomin was able to attenuate O_2^- production in both diabetic and WD rat aortae. The acute exposure of aortae to α-tocopherol and tocomin was able to significantly improve endothelium-dependent relaxation in both diabetic and WD rats which was the hypothesis of the study (Hypothesis 3). Similarly to Chapter, 3 tocomin was up to 100 times more potent in improving endothelium dependent relaxation in both diabetic and western-diet fed rat aortae. Endothelium-dependent relaxation in the WD aortae is almost entirely mediated through eNOS derived NO however the possible mechanism through which acute exposure of the WD aorta to tocomin improves endothelium-dependent relaxation is through a non-eNOS source of NO or a greater contribution of EDHF-type relaxation which was seen as an enhanced maximum relaxation response of the WD rat aorate in the presence of L-NAME plus tocomin in comparison to L-NAME alone.

This study also demonstrated the different mechanisms of endothelial dysfunction in the diabetic and WD rat aortae in comparison to the sham and SD aortae respectively. Apart from increased Nox2 expression that was consistent with increased O_2^- production in the diabetic and WD aortae, both diabetic and WD aortae also had decreased eNOS expression. However, during diabetes decreased eNOS expression is not due to decreased pAkt/Akt-induced eNOS activation or altered CaM/cav-1 expression the expression of these proteins was not affected during diabetes. Rather these results suggest that in advanced diabetes (10 weeks), the potential up-regulation of other O_2^- producing pathways e.g. polyol pathway and mitochondrial activity may

cause uncoupling of eNOS thus further exacerbating endothelial dysfunction. In contrast in the WD aorta, decreased eNOS activity is due to decreased Akt-induced eNOS activation that was demonstrated by a decreased pAkt/Akt ratio, a decrease in the eNOS stimulatory protein CaM expression and an increase in the eNOS inhibitory cav-1 expression.

6.3 The effect of 4-week tocomin treatment in rat aortae animal models of diabetes and obesity.

Having demonstrated the ability of tocotrienol rich tocomin to attenuate oxidative stress and improve vascular function in the presence of pyrogallol-induced oxidative stress and diabetic and obese rat aortae *in vitro* the aim of the final study was to investigate whether the treatment of diabetic and high-fat fed rats with tocomin can improve endothelial function in the aortae and improve the expression of eNOS and decrease the expression of the O_2^- producing enzyme Nox2 (Aims 4&5).

We demonstrated that 4-week treatment with the tocotrienol-rich extract of palm oil, tocomin (40 mg/kg/day s.c.), increases NO activity to improve endothelium-dependent relaxation in aortae from diabetic rats and rats fed a high-fat "western" diet (Hypothesis 4). Tocomin did not affect the diet-induced weight gain or increase in epididymal fat but did attenuate the vascular oxidative stress. Our previous study demonstrated that tocomin, which contains a high proportion of tocotrienols (48%) with some α-tocopherol (11%), is able to acutely reduce oxidative stress to improve endothelium-dependent relaxation in vitro (Chapter 3). An additional beneficial action in this study to reduce vascular oxidative stress was a decreased expression of the vascular NADPH oxidase subunit Nox2 which is perhaps due to tocomin acting at a cellular level

decreasing PKC-induced Nox2 activation, however this requires further investigation. A further positive outcome of tocomin treatment in the diabetic and obese rats was an increased expression of eNOS (Hypothesis 5) and its positive regulatory proteins calmodulin and pAkt/Akt. This study also revealed that in the WD aortae, eNOS expression is promoted by the proteins calmodulin and pAkt, whereas in the diabetic aortae endothelial function may potentially be improved due to an increase in eNOS coupling which was seen as a signifiacant improvement in eNOS expression. Further, there was also a decreased expression of the inhibitory protein caveolin-1. The beneficial actions of tocomin observed in these diet-induced model of diabetes and obesity suggest that it may have potential to be used as a therapeutic agent to prevent vascular disease in diabetes and obesity.

6.4 Future directions

This thesis has extended upon the clear connection between type 1 diabetes and obesity and its ability its cause oxidative stress and subsequent endothelial dysfunction. The ability of tocotrienol rich tocomin to alleviate oxidative stress and improve endothelial dysfunction has also been made in this thesis. Therefore, based on the findings from this thesis, studies to further investigate the function of tocotrienols might include;

1. Investigating the expression of the eNOS protein as monomer/dimer and its level of phosphorylation at Thr495 and S1177 to further assess the effect of diabetes, a high- western diet and tocomin treatment on eNOS state of activation.

2. Investigating the effect of tocomin on endothelial dysfunction and oxidative stress in the same animal models of diabetes and obesity in the microvasculature

(eg: cerebral and mesenteric arteries) as an assessment in resistance rather than conductance vessels.

3. Investigating the effect of tocomin on endothelial dysfunction and oxidative stress in a type 2 diabetes animal model such as the Zucker rat in the macro- (e.g. aorta) and micro-vasculature (e.g. mesentery artery). Of particular interest is how obesity and type 2 diabetes affects eNOS phosphorylation (Ser1179/1177 and Thr495) and eNOS coupling and the effect of tocomin on eNOS phosphorylation and eNOS coupling.

4. The effect of type 2 diabetes on inflammatory markers of obesity (e.g. C-reactive protein, TNF-α and IL-6) and insulin levels and whether tocomin can decrease the expression of any of these markers or affect insulin levels. Of interest will also be the effect of type 2 diabetes on lipid adiponectin levels and whether tocomin can affect adiponectin levels.

5. Other areas of possible investigation would be the effect of obesity and type 2 diabetes and its effect on pancreatic β-cell function and expression. Also studying fasting insulin levels to determine the presence of insulin resistance and investigate whether tocomin treatment has any effect on pancreatic β-cell function.

6.5 Conclusion

There is a significant body of evidence gathered from both animal and human studies that ROS play a fundamental role in CVD (Griendling and Fitzgerald, 2003) including in disease associated with diabetes and obesity (Furukawa et al., 2004). It is logical then that there has been intensive investigation of antioxidants in the prevention and treatment of cardiovascular disease, but despite many positive results in animal studies, the outcomes of large clinical trials have been predominantly disappointing (Schmidt et al., 2015). These include extremely high concentrations of tocopherol required to achieve any physiologically beneficial outcome and tocopherol becoming a prooxidant at high concentrations. Studies examining the potential benefits of vitamin E have predominantly focused on α-tocopherol, one of eight naturally occurring isoforms of that vitamin, while there has been relatively little investigation of the potential capacity of tocotrienols as therapeutic agents (Peh et al., 2016). There is growing evidence that tocotrienols do not simply mimic the biological actions of tocopherols. For example, tocotrienols are reported to more effectively accumulate in cells compared to tocopherols, and this has been suggested to promote their efficacy as antioxidants (Saito et al., 2004). Further, we have demonstrated that the combination of tocotrienols with α-tocopherol is better able to preserve endothelial function in the presence of oxidative stress than either tocopherol or tocotrienols alone (Chapter 3). This could possibly be due to tocopherols enhancing the antioxidant activity of tocotrienols or the presence of α-tocopherol is necessary for the potent antioxidant activity of tocotrienols due to the high biological affinity of α-tocopherol to the α-TTP.

The outcome of the first study, where a tocotrienol-rich (48%) extract that includes some α-tocopherol (11%) preserved endothelial function in the presence of pyrogallol-induced oxidative stress, suggests further investigation of tocomin as a potential therapeutic agent is warranted. We further demonstrated that the tocotrienol-rich extract of palm oil, tocomin, acutely improves endothelium-dependent relaxation and increases NO activity to improve endothelium-dependent relaxation in aortae from diabetic and obese rats fed a high-fat "western" diet (Chapter 4). An additional beneficial action in this study to reduce vascular oxidative stress was a decreased expression of the vascular NADPH oxidase subunit Nox2 in both diabetic and WD rat aortae. This could possibly be due to reduced oxidative stress in the aortae resulting from the antioxidant affect of tocomin having a downstream effect on Nox2 expression. A further positive outcome of tocomin treatment in the diabetic and obese rats was an increased expression of eNOS in the diabetic and obese rat aortae which could possibly due to due the decreased O_2^- production in the vasculature increasing the bioavailability of eNOS cofactors such as BH_4 from being reduced thus maintaining eNOS integrity. Also decreased O_2^- production may also prevent the uncoupling of eNOS due to a decrease in oxidative stress, however this requires further investigation. In the WD aortae eNOS activity promoting proteins calmodulin and pAkt expression was improved. Further, there was also a decreased expression of the inhibitory protein caveolin-1. Interestingly, neither diabetes nor tocomin affected the proportion of phosphorylated Akt to Akt and the expression of calmodulin and caveolin-1.

The beneficial actions of tocomin in these models of diabetes and diet-induced of obesity suggest that it may have potential to be used as a therapeutic agent to prevent vascular disease in diabetes and obesity. This thesis has made a significant contribution

to the field of endothelial dysfunction and the pharmacology of vitamin E which has resulted in a better understanding of the effects of tocotrienols on endothelial dysfunction in animal models of diabetes and obesity. This was necessary to study to determine whether these compounds may be able to create new adjunct therapy for diabetes and obesity induced cardiovascular disease

Chapter 7

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CHAPTER 7: REFERENCES

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Appendix

Original

AEC 09/08
Recd 22/9/08

RMIT ANIMAL ETHICS COMMITTEE

APPLICATION FOR APPROVAL TO USE ANIMALS IN A RESEARCH OR TEACHING PROJECT

Date received *Office Use Only*

School

AEC Register Number

0	8	2	2	

Dbox 7/10/08

CONDITIONS OF APPROVAL

All matters pertaining to the conduct of the approved project are to be reported to the RMIT Animal Ethics Committee, which maintains oversight in accordance with licence conditions for the RMIT Licences SPPL 49, SPPL 50, SPPL 302 and SPFL 40.

Any variation proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until approval is granted.

A record of details of any animals used in the project must be retained.

The project should only be conducted in approved premises nominated on the Licence SPPL 49. Use of other premises would constitute a variation and relevant details are to be notified to the AEC for approval as "field work".

The AEC must also be notified of:

- Any changes to approved investigators
- Any incidents involving animals used in the project and the steps taken to deal with them.

A report on this project must be provided to the AEC at the end of each calendar year.

The total numbers of animals approved for use in the project are:

Species (and common name)	Strain [indicate (*) if Genetically Modified]	Sex	Age	Total Number
Rat	Wistar	Male	8 weeks	280

DECLARATION BY NOMINATED SIGNING OFFICER FOR THE AEC

I certify that this project has been considered and approved by the RMIT Animal Ethics Committee

The period of approval for the project is 13 / 10 /20 08. to 30 / 09 /2011

Name of Chair	**A/Prof Karen Nankervis**
Signatu..:	
Date:	**2/10/2008**

COPY PROVIDED TO INVESTIGATOR ON ... 7/10/08 .....

Original

RMIT ANIMAL ETHICS COMMITTEE

APPLICATION FOR APPROVAL TO USE ANIMALS IN A RESEARCH OR TEACHING PROJECT

Date received
Office Use Only
13/5/10.

School

AEC Register Number

	1	0	1	1

CONDITIONS OF APPROVAL

All matters pertaining to the conduct of the approved project are to be reported to the RMIT Animal Ethics Committee, which maintains oversight in accordance with licence conditions for the RMIT Licences SPPL 49(Medical Sciences), SPPL 50(Applied Sciences), SPPL 302(RDDT), SPPL 365(Health Sciences) and SPFL 40.

Any variation proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until approval is granted.

A record of details of any animals used in the project must be retained.

The project should only be conducted in approved premises nominated on the Licence **SPPL 49.** Use of other premises would constitute a variation and relevant details are to be notified to the AEC for approval as "field work".

The AEC must also be notified of:

- Any changes to approved investigators
- Any incidents involving animals used in the project and the steps taken to deal with them.

A report on this project must be provided to the AEC at the end of each calendar year.

The total numbers of animals approved for use in the project are:

Species (and common name)	Strain [indicate (*) if Genetically Modified]	Sex	Age	Total Number
Rat	Sprague Dawley	M	6-10 weeks	540

DECLARATION BY NOMINATED SIGNING OFFICER FOR THE AEC

I certify that this project has been considered and approved by the RMIT Animal Ethics Committee

The period of approval for the project is 1 / 6 /2010 to 31 / 5 /2013 .

Name of Chair	**Prof Stephen Bird**
Signature.	[redacted]
Date:	**13/5/2010**

COPY PROVIDED TO INVESTIGATOR ON ... 13/5/10 .....

Original

RMIT ANIMAL ETHICS COMMITTEE

APPLICATION FOR APPROVAL TO USE ANIMALS IN A RESEARCH OR TEACHING PROJECT

Date received
Office Use Only

School

AEC Register Number

	1	1	2	1

CONDITIONS OF APPROVAL

All matters pertaining to the conduct of the approved project are to be reported to the RMIT Animal Ethics Committee, which maintains oversight in accordance with licence conditions for the RMIT Licences SPPL 49(Medical Sciences), SPPL 50(Applied Sciences), SPPL 302(RDDT), SPPL 365(Health Sciences) and SPFL 40.

Any variation proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until approval is granted.

A record of details of any animals used in the project must be retained.

The project should only be conducted in approved premises nominated on the Licence **SPPL 49.** Use of other premises would constitute a variation and relevant details are to be notified to the AEC for approval as "field work".

The AEC must also be notified of:

- Any changes to approved investigators
- Any incidents involving animals used in the project and the steps taken to deal with them.

A report on this project must be provided to the AEC at the end of each calendar year.

The total numbers of animals approved for use in the project are:

Species (and common name)	Strain (indicate (*) if Genetically Modified)	Sex	Age	Total Number
Rat	Wistar	Male	8 weeks	240

DECLARATION BY NOMINATED SIGNING OFFICER FOR THE AEC

I certify that this project has been considered and approved by the RMIT Animal Ethics Committee

The period of approval for the project is 1/9/20 11 **to** 31/8/20 14

Name of Chair	A/Prof Peter Smooker
Signature:	
Date:	1/8/2011

COPY PROVIDED TO INVESTIGATOR ON ... 1/8/2011 ...

RMIT UNIVERSITY

Research & Innovation

GPO Box 2476V
Melbourne VIC 3001
Australia

Tel. +61 3 9925 2251
Fax +61 3 9925 6599

5 April 2012

Dr Trisha Jenkins
School of Medical Sciences
RMIT University

Dear Trisha

AEC Project No. 1211: Western diet and its effect on learning, memory and motivation in rats.

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from **5 April 2012** until **31 March 2015**. An approved version of the application is attached.

The use of animals in scientific procedures is strictly regulated by the *Australian code of practice for the care and use of animals for scientific purposes* (the 'Code'). The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare. There are several aspects of the Code, the license conditions and the operations of the AEC that I would like bring to your attention.

Responsibilities of investigators
Responsibilities of investigators are described in the *Australian code of practice for the care and use of animals for scientific purposes* (section 3). According to the Code investigators have a 'personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the Code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project' (s.3.1.1).

Amendments and extensions
If as you proceed with your project you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the Committee may only deal with 'minor' amendment requests. Major amendments to projects normally require a new project application.

Minor amendments including the addition of staff to a project or requests for time extensions can be reviewed by an executive between meetings if necessary, otherwise requests are dealt with at the regular scheduled meetings of the AEC. If executive approval is required please contact the secretary directly.

Time extensions are normally granted for projects, but cannot be granted retrospectively in any circumstances.

Adverse events or unexpected outcomes
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the Committee immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.

RMIT UNIVERSITY

RMIT UNIVERSITY ANIMAL ETHICS COMMITTEE (AEC)	
Application For Approval To Use Animals In A Research Project	**Date application received:**

Office Use Only

Project Title AEC Register Number

Understanding how western diet induced weight gain affects learning and memory

AEC Permit Number

1	2	4	5	

DECLARATION BY AEC CHAIRMAN

I certify that this project has been considered and approved by the RMIT University AEC on the _____

The period of approval for this project is 1/2/12 **to** 31/1/16

AEC Chairman Name:	David Glanz
AEC Chairman Signature:	
Date:	6/12/2012

CONDITIONS OF APPROVAL

All matters pertaining to the conduct of the approved project are to be reported to the AEC, which maintains oversight in accordance with licence conditions for the Licence **SPPL - [insert license number]**. (449)

Any variation proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until approval is granted.

A record of details of any animals used in the project must be retained.

The project should only be conducted in approved premises nominated on the Bureau of Animal Welfare Scientific Licence **SPPL - [insert license number]**. 449

The AEC must also be notified in writing of;

- Any changes to approved investigators
- Any unexpected incidents or complications that result in deaths, euthanasia or pain and suffering for the animals used in the project. Details of the steps taken to deal with adverse incidents must be included in the notification.

OTHER CONDITIONS:

This approval is subject to the following special conditions;

RMIT UNIVERSITY

AEC 01/13

RMIT UNIVERSITY ANIMAL ETHICS COMMITTEE (AEC)	
Application For Approval To Use Animals In A Research Project	Date application received:

Office Use Only

Project Title AEC Register Number

AEC Permit Number

1	3	0	9	

DECLARATION BY AEC CHAIRMAN

I certify that this project has been considered and approved by the RMIT University AEC on the

The period of approval for this project is 1/3/13 to 28/2/15

AEC Chairman Name:	David Story
AEC Chairman Signature:	
Date:	7/2/2013

CONDITIONS OF APPROVAL

All matters pertaining to the conduct of the approved project are to be reported to the AEC, which maintains oversight in accordance with licence conditions for the Licence SPPL - [insert license number]. 49

Any variation proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until approval is granted.

A record of details of any animals used in the project must be retained.

The project should only be conducted in approved premises nominated on the Bureau of Animal Welfare Scientific Licence SPPL - [insert license number]. 49

The AEC must also be notified in writing of;

- Any changes to approved investigators
- Any unexpected incidents or complications that result in deaths, euthanasia or pain and suffering for the animals used in the project. Details of the steps taken to deal with adverse incidents must be included in the notification.

OTHER CONDITIONS:

This approval is subject to the following special conditions;

Note: Use of modified monitoring form (see attached).

RMIT UNIVERSITY

Research & Innovation

GPO Box 2476V
Melbourne VIC 3001
Australia

Tel. +61 3 9925 2251
Fax +61 3 9925 6599

24 July 2014

Professor Owen Woodman
School of Medical Sciences
RMIT University

Dear Owen,

AEC 1417: The effect of tocotrienol treatment on pathologies caused by a western diet in rats.

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from **24 July 2014** until **23 July 2017**. An approved version of the application is attached.

Animals
Your application has been approved to use **n=40 rats (Wistar Hooded or Long Evans)** over the duration of the project.

The use of animals in scientific procedures is strictly regulated by the *Australian code of practice for the care and use of animals for scientific purposes*. The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare.

Responsibilities of investigators

1. **Professor Owen Woodman**
2. **Dr Trisha Jenkins**
3. **Ms Saher Ali**
4. **Mr Jason Nguyen**

Responsibilities of investigators are described in the *Australian code of practice for the care and use of animals for scientific purposes* (section 3). Investigators have a 'personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project' (s.3.1.1).

Amendments and extensions
If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with 'minor' amendment requests. Major amendments to projects normally require a new project application.

Adverse events or unexpected outcomes
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.